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TESIS DOCTORAL

Variabilidad morfológica y nucleotídica en el género "cistus" : análisis macro- y microevolutivos

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VARIABILIDAD MORFOLÓGICA Y NUCLEOTÍDICA EN EL GÉNERO *CISTUS*: ANÁLISIS MACRO- Y MICROEVOLUTIVOS

TESIS DOCTORAL

BEATRIZ GUZMÁN ASENJO
Madrid, 2008

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Gracias

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Introducción

1. Macro- y microevolución, ¿cuestión de grado, de tipo o ambos?

"*Natura non facit saltum.* Como la selección natural obra solamente por acumulación de variaciones favorables, pequeñas y sucesivas, no puede producir modificaciones grandes o súbitas; puede obrar solamente a pasos cortos y lentos" (Darwin 1859). Con la publicación de El origen de las especies, Darwin abrió un debate sobre procesos macro- y microevolutivos que hoy en día todavía suscita controversia (Erwin 2000; Carroll 2001; Simons 2002).

Por **macroevolución** se entiende la diferenciación de organismos por encima del nivel de especie (géneros, familias, órdenes y *phyla*), descritos en sistemática (Stearns & Hoekstra 2001). Mientras que **microevolución** atiende a organismos por debajo del nivel de especie (poblaciones). Los mecanismos responsables del cambio microevolutivo son analizados en todas sus modalidades (mutación, recombinación, flujo genético, deriva, selección natural). Sin embargo para explicar fenómenos de diferenciación a gran escala en el origen de las mayores novedades evolutivas (como la existencia de gradientes latitudinales de diversidad, extinciones masivas, formación súbita de nuevos fenotipos) se han propuesto distintos mecanismos a lo largo de la historia de la biología: ortogénesis, macromutaciones, especiación "cuántica", equilibrio puntuado y selección de especies (Grantham 2007).

Carroll (2001) apunta que la falta de consenso que, desde tiempos de Darwin, existe a la hora de establecer los dominios de la micro- y de la macroevolución deriva de la concepción diferente que paleontólogos y genéticos de poblaciones tienen de esta última. Mientras que para los primeros se trata de una evolución filética (origen y extinción de poblaciones y especies, tal y como muestra el registro fósil), para los segundos macroevolución es sinónimo de acumulación en el tiempo de secuencias repetidas de microevolución.

Hoy en día se discuten tres hipótesis para establecer la relación existente entre los dominios de la macro- y de la microevolución (Grantham 2007). La primera y la tercera surgen en el seno del neo-darwinismo, dividiendo esta corriente en dos ramas:

ultradarwinistas y naturalistas. La segunda hipótesis es defendida por paleontólogos y otros macroevolucionistas, quienes advierten de la existencia de discontinuidades entre los procesos microevolutivos y los patrones a gran escala detectados en el registro fósil, que hacen que ciertos mecanismos microevolutivos que describen correctamente la evolución a nivel poblacional sean totalmente inadecuados para explicar ciertos casos de evolución a gran escala. Del neo-darwinismo se desprende que si la acción de la selección natural es continua y prolongada, debería existir entre dos especies emparentadas una secuencia de formas intermedias que unieran gradualmente la forma ancestral con las derivadas (gradualismo filético). Si bien es cierto que en algunos linajes fósiles se puede establecer una continuidad bien documentada entre micro- y macroevolución por el hallazgo de formas intermedias (e.g. Ahlberg *et al.* 1996; Zimmer 1998; Sage & Monson 1999; Rubidge & Sidor 2001; Zhou & Zheng 2003), también es cierto que el registro fósil sigue presentando discontinuidades para otros muchos linajes.

Las relaciones que se establecen hoy en día entre el dominio de la macro- y de la microevolución son las siguientes:

1. Explicación reduccionista de la macroevolución. Basada en el reduccionismo (Sarkar 1998), defiende que la macroevolución no es más que la suma de procesos microevolutivos de modo que, con una escala temporal adecuada, la microevolución, por sí sola, podría explicar los procesos macroevolutivos (Vermeji 1987, 1994). Según esta hipótesis la especiación conectaría la macro- y la microevolución, y todos los fenómenos de microevolución tendrían algún efecto por encima del nivel de especie (en tanto en cuanto que los niveles superiores de la clasificación Linneana son artificios construidos por los sistemáticos para su conveniencia).

2. Explicación no reduccionista de la macroevolución. La naturaleza puntuada de parte de los procesos de especiación (Jackson 1995; Jackson & Cheetham 1999), las extinciones masivas (Jablonski 2005) y la aparición de novedades evolutivas no azarosas (Kendrick & Crane 1997; Valentine *et al.* 1999) dan lugar a una acción jerarquizada de la

evolución que impide y neutraliza los efectos de la microevolución (Erwin 2000). Según esta hipótesis existen mecanismos macroevolutivos diferentes e independientes a los microevolutivos que actúan sobre propiedades emergentes de las especies (como el rango geográfico o la estructura genética de las poblaciones) y niveles taxonómicos superiores (linajes de especies, clados, comunidades ecológicas, etc). El mecanismo macroevolutivo por excelencia sería la selección de especies (Grantham 2007; Jablonski 2007), de manera que las perturbaciones en el ambiente también controlarían los patrones de reemplazamiento de clados, cambios en las comunidades y la especiación. En este sentido, se ha comprobado que un mayor rango geográfico estaría correlacionado negativamente con el riesgo de extinción y la tasa de especiación (Jablonski 1987; Gaston & Chown 1999; Purvis *et al.* 2000; Jablonski & Roy 2003; Jones *et al.* 2003; Jablonski & Hunt 2006; Grantham 2007), sin embargo el desafío es identificar propiedades emergentes de una forma consistente y analizar empíricamente su papel en diferentes niveles jerárquicos.

3. Explicación intermedia. Los macropatrones pueden ser explicados mediante combinaciones complejas de procesos ocurridos en niveles superiores e inferiores, de manera que ambos tipos de evolución (macro- y micro-) actuarían simultáneamente en distintos niveles.

La **biología evolutiva del desarrollo** (Evo-Devo por sus siglas en inglés, *Evolutionary Developmental Biology;* Hall 1999) despunta con fuerza como disciplina que trata de identificar, entre otras cosas, los mecanismos del desarrollo que ocasionan cambios evolutivos en el fenotipo de los organismos (Gould 1977; Hall & Olson 2003). La importancia que está adquiriendo la Evo-Devo reflejaría el empeño en encontrar y comprender la relación existente entre la transformación ocurrida en un organismo en una única generación (desarrollo, ontogenia, cambios ontogénicos) y las ocurridas entre generaciones (evolución, filogenia, cambios filogenéticos). La combinación de la biología evolutiva del desarrollo y la genética molecular que se produce en la Evo-Devo podría arrojar luz sobre los mecanismos que hay detrás de la macroevolución y así lo

demuestra uno de sus principios más importantes: cambios en el control de los genes de desarrollo (más que modificaciones de genes estructurales) deben ser una de las principales causas de los cambios evolutivos morfológicos más notables (Theissen & Saedler 1995; Doebley & Lukens 1998).

La macroevolución se ha estudiado principalmente desde un punto de vista zoocéntrico, ignorando que el registro fósil de plantas terrestres y otros organismos fotosintéticos es amplísimo. La Evo-Devo está poniendo de manifiesto que el estudio únicamente en animales no permitirá detectar todos los patrones macroevolutivos (Theissen *et al.* 2000), en tanto en cuanto se hace necesaria la comparación de los patrones evolutivos de linajes primigenios que provienen de ancestros unicelulares independientes. Esta condición que cumple la comparación del reino protista, animal y vegetal hace necesaria la realización de estudios en organismos vegetales, entre otros. En esta dirección se están llevando a cabo numerosas investigaciones acerca de la evolución floral a través del estudio de los genes MADS-box (Theissen *et al.* 2000; Irish 2003) y, en concreto, del modelo ABC de identidad de órganos florales (Coen & Meyerowitz 1991; Angenent & Colombo 1996; Pelaz *et al.* 2000) en especies modelo (*Arabidopsis thaliana, Antirrhinum majus, Petunia hybrida, Nicotiana tabacum, Oryza sativa,* y *Zea mays*). Las especies modelo son una herramienta fundamental en algunas áreas de la investigación científica pues permiten identificar el nivel de conocimiento a partir de unas pocas especies. Sin embargo, para determinar el papel evolutivo de los mutantes homeóticos sería necesario el estudio de poblaciones naturales (*Clarkia concinna*, Ford & Gottlieb 1992; *Linaria vulgaris*, Cubas *et al.* 1999; *Capsella bursa-pastoris*, Hintz *et al.* 2006), puesto que el establecimiento de nuevos linajes a partir de estos mutantes requiere de su supervivencia durante años bajo los efectos de la selección natural.

Por otro lado, la importancia que está adquiriendo el papel del ambiente en la evolución ha originado un "boom" en la **epigenética** (disciplina que estudia los cambios heredables en la función génica del ADN que se producen sin variar su secuencia nucleotídica), pues actuaría como puente entre los efectos genéticos y los ambientales. Los ejemplos sobre cambios epigenéticos heredables son ya significativos en la

literatura (Meyer *et al.* 1992; Das & Messing 1994; Cubas *et al.* 1999). Muchos autores han discutido el papel de la epigenética en la evolución, destacando que la perspectiva epigenética puede ser más informativa que la genética "convencional" a la hora de explicar ciertas cuestiones evolutivas relativas a la ontogenia, organización del genoma y especiación (Jablonka & Lamb 1998). En este sentido, Newman & Müller (2000) sugieren que mecanismos epigenéticos, más que cambios genéticos, han sido las principales fuentes de innovaciones morfológicas en la evolución (macroevolución).

Otro fenómeno que pone de manifiesto que las interacciones genotipo-ambiente han debido y deben jugar un papel fundamental en la macroevolución es la **plasticidad fenotípica** (Pigliucci 2001). Definida como el conjunto de fenotipos alternativos expresados por un mismo genotipo en respuesta a señales ambientales (Schlichting & Smith 2002), la plasticidad que ha evolucionado para producir formas morfológicas distintas en diferentes estaciones, situaciones de estrés o en respuesta a distintos tipos nutricionales ha de verse como un mecanismo de evolución rápido capaz de ayudarnos a entender mecanismos macroevolutivos como la evolución de novedades fenotípicas, fenómenos de especiación y colonización de nuevos nichos ecológicos. La selección natural actuaría sobre fenotipos alternativos y por tanto sobre el genotipo que los posibilita. Algunos ejemplos de la relevancia macroevolutiva de la plasticidad fenotípica son la determinación del sexo por la temperatura, no solo en insectos sino también en reptiles y otros vertebrados, y su relación con el genotipo (Crews 1994). La hipótesis del origen del viviparismo en reptiles por mejora del fenotipo de la descendencia debido a exposición del huevo a altas temperaturas (Shine 1995), más que por cuestiones de protección como apunta la hipótesis clásica, es también esgrimida.

Estos tres campos de investigación (Evo-Devo, epigenética y plasticidad fenotípica) ponen de manifiesto otro debate abierto sobre la necesidad de una nueva síntesis evolutiva que permita integrar los conceptos e información obtenida de disciplinas como la biología molecular del desarrollo, la epigenética, la ecología, la sistemática, la geología y la paleontología (Carroll 2000; Sandvik 2000; Kutschera & Niklas 2004). Un ejemplo de integración de las bases genéticas de la evolución morfológica, la sistemática

y la ecología es el trabajo de Hileman *et al.* (2003) sobre la simetría floral y su efecto en los mecanismos de polinización. En dicho trabajo se estudiaron dos especies (*Mohavea confertiflora* y *Antirrhinum majus*) que muestran grandes diferencias en el desarrollo y forma floral. Tradicionalmente segregadas en dos géneros, estudios moleculares recientes han puesto de manifiesto que la flor de *Mohavea* debe ser derivada de una flor similar a la que posee *Antirrhinum* (*Mohavea* y ciertas especies americanas de *Antirrhinum* se encuentran en el mismo clado). El estudio pone de manifiesto que cambios en los patrones de expresión de los genes responsables de la simetría floral (CYCLOIDEA y DICHOTOMA) dieron lugar a una forma floral adaptada a la polinización por abejas colectoras de polen en lugar de por abejas robadoras de néctar. De esta forma se pone de manifiesto que una aproximación integradora que unifique la ecología, la genética y el desarrollo tienen el potencial de aumentar nuestro conocimiento acerca de los mecanismos que operan en la evolución adaptativa.

2. Justificación y objetivos

La presente memoria doctoral no pretende explícitamente proponer las causas últimas de procesos macro- y microevolutivos. Por el contrario una serie de experimentos han sido diseñados para reconstruir patrones evolutivos en Cistáceas. El género *Cistus* L. ha sido elegido por presentar uno de sus centros de diversificación en la Península Ibérica (de las 21 especies reconocidas, 12 están presentes en dicha área geográfica) y por los desconocidos mecanismos de diferenciación subyacentes.

La memoria doctoral se divide en dos partes. En la primera parte se realiza un **análisis macroevolutivo** (por encima del nivel de especie) mediante el estudio de las relaciones filogenéticas de representantes de la familia de las Cistáceas y del género *Cistus*. Al inicio de nuestros estudios no existía ningún trabajo publicado que analizara la diversidad genética y evolución de todas las especies de jaras. Dansereau (1939) esbozó un diagrama filético basado en caracteres morfológicos (Fig. 1) en el que las especies de flores púrpuras se disponían en una posición ancestral con respecto a aquellas de flores blancas, particularmente las que presentan tres sépalos. Por otro lado,

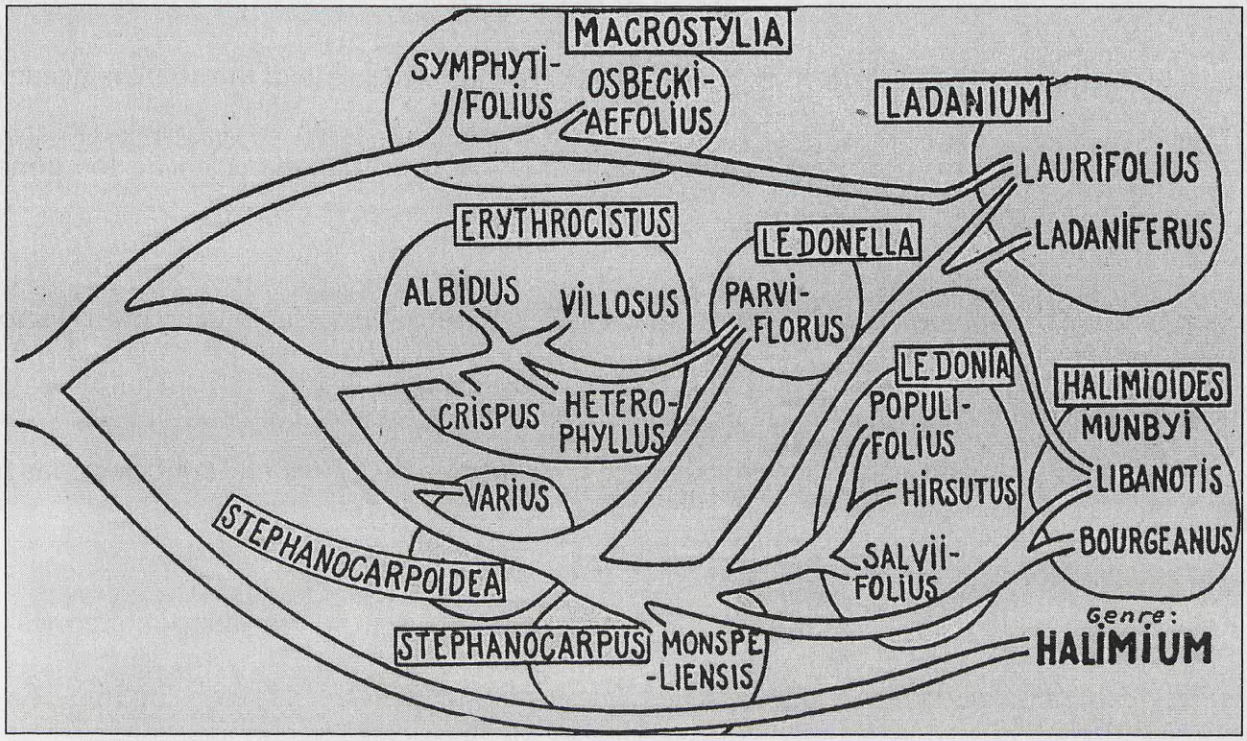


Fig. 1. Diagrama filético propuesto por Dansereau (1939) en su monografía del género *Cistus*

Batista *et al.* (2001) realizaron un estudio de la diversidad genética de tres endemismos canarios (*C.symphytifolius, C. chinamadensis* y *C. osbeckiifolius*), encontrando niveles de diversidad genética altos para la primera especie, medios para la segunda y bajos para la última. Nadie más pareció preocuparse de las relaciones de parentesco entre las 21 especies de jaras.

En la segunda parte de la tesis se lleva a cabo un **análisis microevolutivo** empleando como objeto de estudio una especie de gran diversidad morfológica: *Cistus ladanifer*. En concreto se han realizado análisis filogeográficos y morfológicos.

Atendiendo a las anteriores consideraciones, el objetivo general de la memoria que se presenta es **inferir las relaciones de parentesco en la familia Cistaceae empleando marcadores moleculares, con especial atención a las especies del género *Cistus*; un estudio poblacional sirve para determinar procesos microevolutivos en un marco geográfico. En última instancia se pretende conectar macro- y microevolución.**

A continuación se muestra una relación de los objetivos concretos:

1. establecer las relaciones filogenéticas entre los géneros de la familia Cistaceae,

2. estudiar las relaciones filogenéticas existentes entre las especies de los géneros *Cistus* y *Halimium*,

3. analizar la monofilia de las especies del género *Cistus* e inferir sus relaciones filogenéticas y la evolución de caracteres morfológicos,

4. realizar un estudio filogeográfico de las especies de *Cistus* endémicos de las islas Canarias para:

    4.1. evaluar la monofilia de las especies, estudiando varias poblaciones por especie, y determinar las especies hermanas,
    4.2. describir la estructura poblacional a través del estudio de haplotipos,
    4.3. proponer patrones de colonización seguidos por las especies entre las distintas islas,
    4.4. reconstruir los eventos de divergencia de poblaciones mediante la reconstrucción de tiempos de divergencia,

5. realizar un estudio filogeográfico de *Cistus ladanifer* para:

    5.1. inferir las relaciones de parentesco de las poblaciones en su distribución total,
    5.2. evaluar el efecto de los últimos eventos climatológicos en la diferenciación de las poblaciones de *Cistus ladanifer*,
    5.3. determinar si la distribución actual de las poblaciones de la especie son el resultado de fenómenos de fragmentación (vicarianza) o, si por el contrario, responde a migraciones salvando barreras geográficas (dispersión),

6. explorar la existencia de patrones de variación en pétalos, número de valvas, producción y viabilidad de semillas en *Cistus ladanifer*.

Para cumplir los objetivos expuestos se han desarrollado los siete capítulos siguientes, de los cuales los capítulos 2-5 presentan los resultados de la aproximación macroevolutiva, mientras que los capítulos 6-7 y apéndices 1-3 conforman el estudio microevolutivo. El **capítulo 2** muestra los resultados del estudio filogenético de la familia Cistaceae realizado mediante la secuenciación de dos regiones del ADN plastidial (*rbcL* y *trnL-trnF*). El **capítulo 3** ahonda en las relaciones filogenéticas de las especies del género *Cistus*. Se trata de la primera hipótesis filogenética que se publica del género e incluye reconstrucciones de la evolución de caracteres clave en la sistemática del género (color de pétalo, número de sépalos, longitud del estilo y número de valvas en el fruto). El estudio se realizó mediante secuenciación de ADN nuclear (ITS, del inglés *Internal Transcribed Spacer*) y ADN plastidial (*trnK-matK* y *trnL-trnF*). El **capítulo 4** trata de esclarecer las relaciones filogenéticas existentes entre *Cistus* y *Halimium* para determinar si, tal y como muestran ciertos caracteres morfológicos y el hecho de que especies de ambos géneros puedan dar lugar a especies híbridas, son una única entidad evolutiva. Además en este capítulo se analiza el papel de la radiación adaptativa como proceso responsable del origen de especies dentro de estos dos géneros. Para ello se emplearon 6 regiones de ADN nuclear y plastidial, (ITS, el gen de copia simple que codifica para la Glutamina Sintetasa (ncp*GS*), *trnL-trnF*, *trnK-matK*, *rbcL* y *trnS-trnG)*. El **capítulo 5** desarrolla un estudio filogeográfico de las cinco especies de jaras endémicas de canarias. Se estudiaron los haplotipos obtenidos por secuenciación de las regiones plastidiales *trnS-trnG* y *trnK-matK*. Así mismo, se realizó un análisis comparativo de la radiación ocurrida en los linajes de jaras de flor púrpura de Canarias y del continente mediante el estudio de haplotipos y la estimación de edades de divergencia y tasas de diferenciación. Los mismos marcadores plastidiales fueron empleados para realizar el estudio filogeográfico de *Cistus ladanifer* presentado en el **capítulo 6**. Este estudio y los relojes moleculares estimados fueron empleados para analizar el origen de la distribución disyunta de la especie (vicarianza vs. dispersión a larga distancia). Por otra parte, la condición propia de la jara pringosa de presentar frutos con un número variable de valvas (6-12) nos llevó a realizar un estudio de campo (**capítulo 7**) para explorar cuáles podían ser las pautas de esta variabilidad así como de

la variación en la producción de semillas. Para llevar a cabo el análisis se tuvieron en cuenta distintos niveles (geográfico, ecográfico, temporal, altitudinal, filogeográfico y taxonómico). En el **capítulo 8** se realiza una discusión general de los resultados macro- y microevolutivos y se enumeran las conclusiones más relevantes. Dentro del análisis microevolutivo y con el fin de ahondar en la variabilidad infraespecífica encontrada en ciertos caracteres morfológicos de *C. ladanifer* diversos experimentos de campo, explicados en detalle en tres apéndices, fueron realizados. En el **apéndice 1** se analiza el papel adaptativo de la mácula púrpura que *Cistus ladanifer* var. *maculatus* presenta en la base del pétalo mediante la comparación del *fruit set* y *seed set* de las dos variedades de la especie (var. *maculatus* y var. *ladanifer*). En el **apéndice 2** se muestran los resultados obtenidos del examen exhaustivo de la condición autoincompatible de *C. ladanifer* a lo largo de todo el periodo de floración. Finalmente, en el **apéndice 3** se estudia la viabilidad de las semillas de *C. ladanifer* en distintas escalas: individuo, poblaciones y táxones (variedades).

3. La familia Cistaceae

La familia Cistaceae ha sido objeto de diferentes tratamientos taxonómicos (Tournefort 1718; Linnaeus 1753; Dunal 1824; Spach 1836; Willkomm 1856; Ponzo 1921; Martín Bolaños & Guinea 1949) dirigidos, principalmente, a la reorganización de los géneros. Hay cierto consenso en reconocer ocho géneros (Fig. 2): *Cistus* L., *Crocanthemum* Spach, *Fumana* (Dunal) Spach, *Halimium* (Dunal) Spach, *Helianthemum* Mill., *Tuberaria* (Dunal) Spach, *Hudsonia* L. y *Lechea* Kalm., (Martín Bolaños & Guinea 1949; Arrington & Kubitzki 2003) si bien hay autores (Fernald 1917; Calderón 1992) que incluyen las especies pertenecientes al género *Crocanthemum* dentro del género *Helianthemum* secciones *Spartioides* y *Lecheoides*.

Formada por unas 180 especies, de las cuales más del 50% corresponden al género *Helianthemum* Mill., las cistáceas presentan una amplia distribución geográfica en todo el mundo, en especial en las zonas templadas y subtropicales del hemisferio norte y la región Mediterránea. *Cistus, Halimium, Helianthemum, Fumana* y *Tuberaria* se encuentran

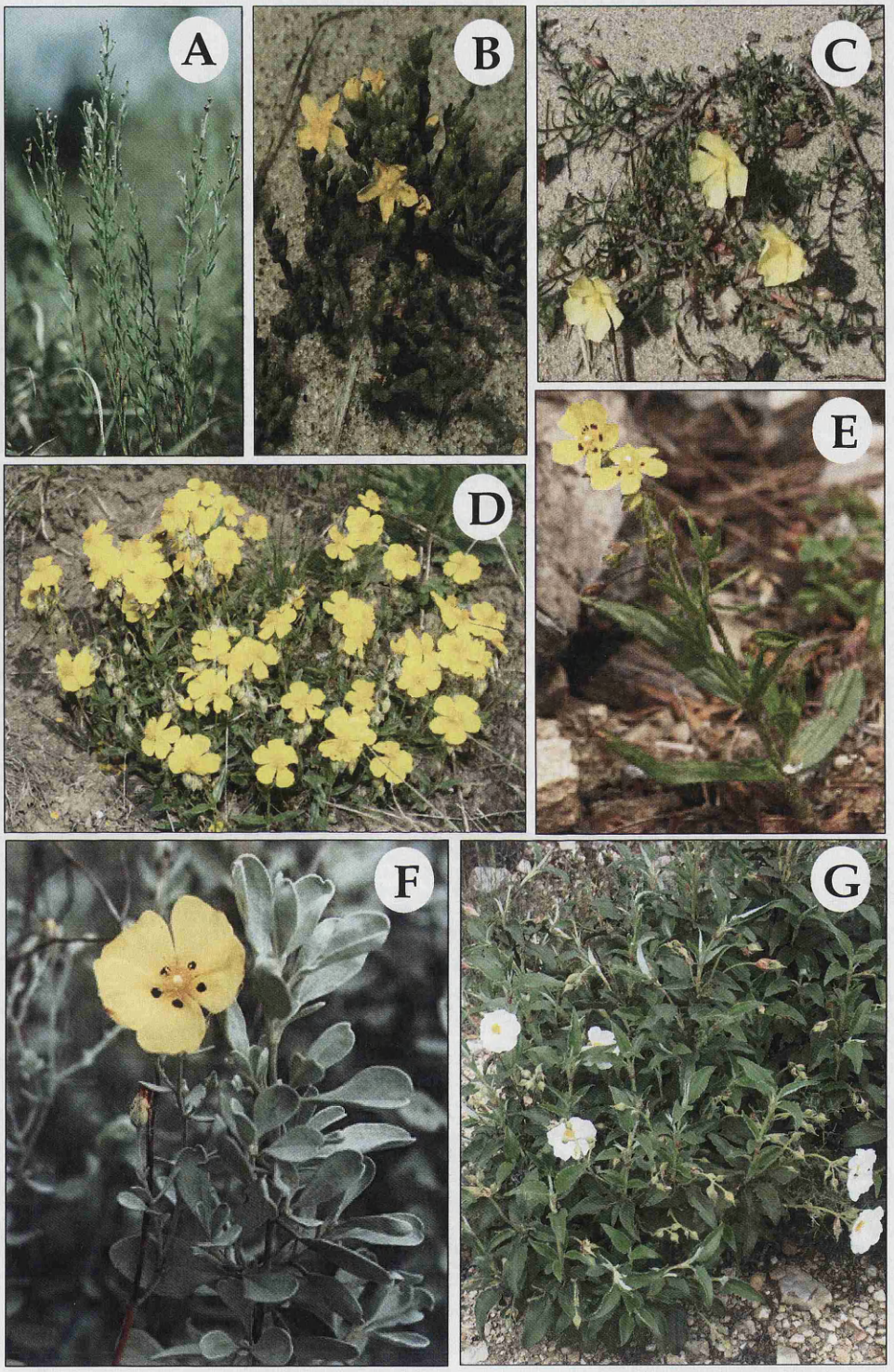


Fig. 2. Representantes de la familia Cistaceae. A, *Lechea stricta* (http://botit.botany.wisc.edu); B, *Hudsonia tomentosa* (George Yatskievych); C, *Fumana procumbens* (http://www.bvnh.de); D, *Helianthemum nummularium* (http://www.sent-online.ch); E, *Tuberaria guttata* (www.wikipedia.org); F, *Halimium halimifolium* (http://crdp2.ac-besancon.fr); G, *Cistus laurifolius* (B. Guzmán).

distribuidos principalmente en la región Mediterránea, si bien hay especies del género *Helianthemum* que se extienden hasta zonas templadas del norte de Europa y zonas del este de China, y especies del género *Cistus* que se extienden hasta las Islas Canarias y el oeste del Sahara. Los géneros *Hudsonia, Lechea* y *Crocanthemum* están presentes en zonas templadas y subtropicales del continente americano.

Dentro de la familia se pueden encontrar arbustos (*C. ladanifer* L.), semiarbustos o sufrútices (*Fumana thymifolia* (L.) Spach ex Webb) y hierbas perennes (*Tuberaria globuraliifolia* (Lam.) Willk.) o anuales (*Tuberaria guttata* (L.) Raf.) con hojas enteras provistas de pelos glandulares, estrellados o simples. Las hojas son opuestas en su mayoría, en tanto que las especies americanas y las especies del género *Fumana* (excepto *F. thymifolia*) presentan casi exclusivamente una disposición alterna. Las especies de los géneros *Cistus, Crocanthemum, Halimium, Hudsonia* y *Lechea* carecen de estípulas, mientras que éstas caracterizan a numerosas especies de los géneros *Helianthemum, Tuberaria* y *Fumana.* Las flores, solitarias o agrupadas en cimas axilares o terminales, son hermafroditas, actinomorfas y están provistas de 3-5 sépalos. Cuando desarrollan 5 sépalos (3 internos y 2 externos), los externos son generalmente más pequeños (a excepción de numerosas especies del género *Cistus,* en las que los sépalos externos son de igual tamaño o mayores que los internos). Únicamente *Lechea* desarrolla flores con 3 pétalos, el resto de géneros presentan flores con 5 pétalos de color blanco, rosa-púrpura o amarillo. Los estambres, numerosos, son todos fértiles, a excepción del género *Fumana* en el que los estambres externos carecen de anteras bien formadas siendo, por tanto, estériles. El gineceo, súpero, está formado por 3 carpelos soldados en un ovario unilocular. Únicamente en el género *Cistus* encontramos ovarios formados por 5 carpelos, siendo una excepción *Cistus ladanifer,* pues presenta un ovario formado por un número variable de carpelos (6-12). El estilo se presenta en diferentes longitudes, dentro incluso del mismo género, tratándose, por tanto, de un carácter de gran significado evolutivo. El fruto es siempre una cápsula que se abre en 3-12 valvas por dehiscencia loculicida. Las semillas son generalmente numerosas y pequeñas, si bien el género *Fumana* presenta semillas gruesas y en número menor. Además suelen ser redondeadas

o poliédricas y no presentan estructuras que faciliten su dispersión. La figura 3 muestra los caracteres diagnóstico de cada uno de los géneros de la familia.

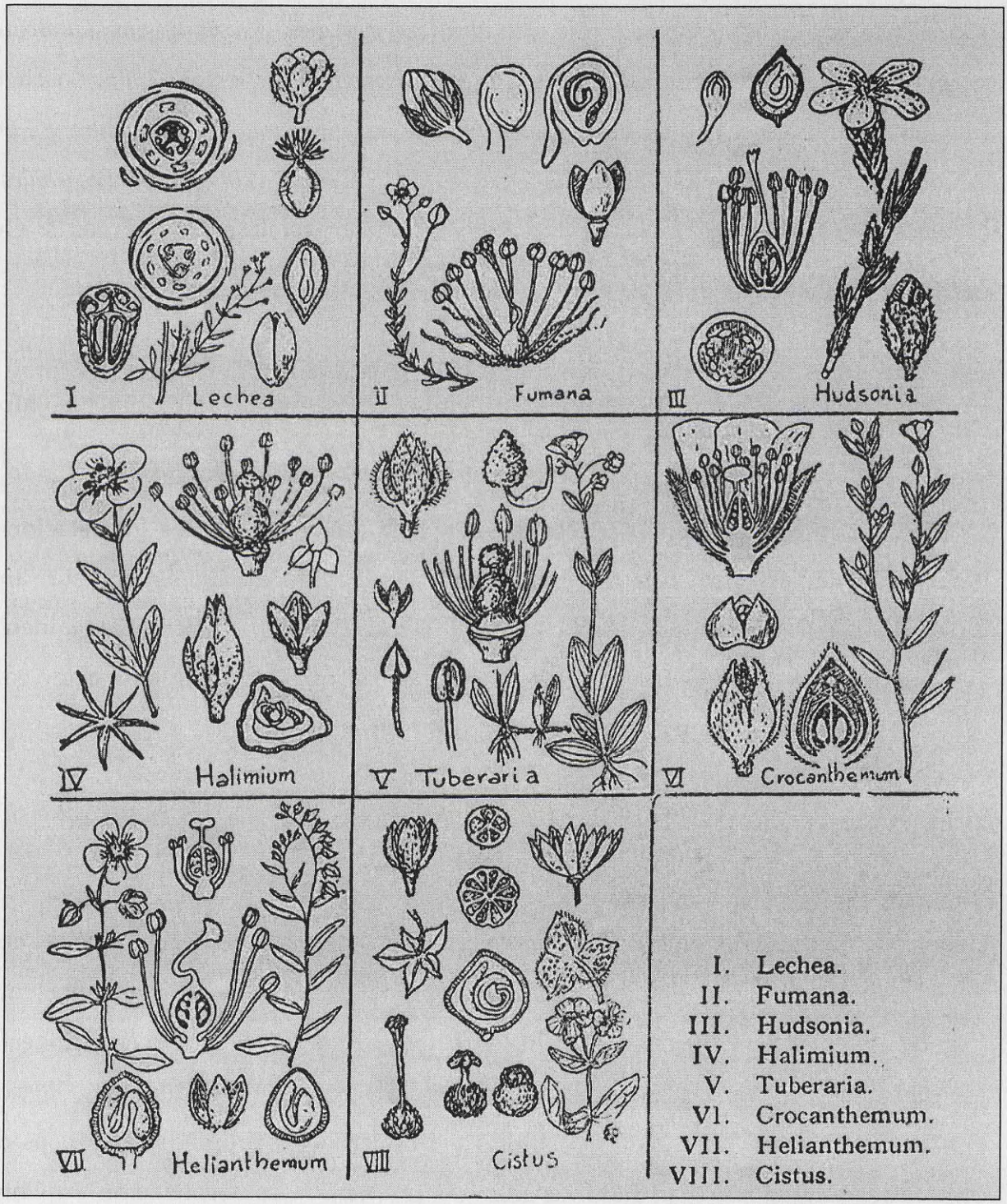


Fig. 3. Representación gráfica de los caracteres diagnóstico de los géneros incluidos en la familia Cistaceae (Martín Bolaños & Guinea 1949).

Dentro de las cistáceas encontramos diferentes sistemas reproductivos, aunque la alogamia es predominante (Herrera 1987; Brandt & Gottsberger 1988; Bosch 1992). Se ha observado en ciertas especies de *Cistus* una completa autoincompatibilidad (Herrera 1987; Talavera *et al.* 1993) mientras que en otras especies (*C. salviifolius, C. albidus*) el nivel de autoincompatibilidad varía según las poblaciones (Herrera 1987; Bosch 1992). La cleistogamia es ocasional en especies de *Cistus, Tuberaria, Helianthemum* y *Fumana,* mientras que es muy habitual en las especies de *Crocanthemum* sección *Lecheoides* y en especies desertícolas de *Helianthemum* sección *Eriocarpum* (Martín Bolaños & Guinea 1949).

Son frecuentes las formas híbridas entre especies de los géneros *Helianthemum, Halimium* y *Cistus* (Gard 1910, 1912, 1914; Demoly 1996), encontrándose incluso híbridos intergenéricos entre especies de *Cistus* y *Halimium* (*Halimiocistus*).

Desde Linneo no ha habido consenso a la hora de establecer las relaciones de parentesco de las Cistáceas con otras familias de plantas. Recientemente, análisis moleculares basados en la secuenciación de ADN (Chase & al. 1993; Savolainen *et al.* 2000) han situado a las Cistáceas dentro del orden Malvales, formando un clado con dos familias tropicales, Dipterocarpaceae y Sarcolaenaceae.

4. El género *Cistus* L.

4.1. Antecedentes históricos

Género de plantas conocido desde antaño, ya es mencionado en la Biblia por el empleo de la resina aromática (llamada ládano) obtenida de algunos de sus representantes (principalmente de *Cistus ladanifer* L., especie curiosamente endémica del oeste del Mediterráneo). Autores griegos y romanos como Herodoto (484-426 adC), Dioscórides (50-70 dC) y Plinio "El Viejo" (23-79 dC) describieron las propiedades y usos de los productos obtenidos a partir de las plantas de *Cistus* y su localización en Chipre y Oriente Próximo.

Sin embargo no es hasta el S. XVI y XVII cuando comienza a haber un interés botánico por la familia de las Cistáceas. Botánicos de la época, como Charles de l'Ecluse, Tabernaemontanus, Barrelier y Bauhin dividieron la familia en dos grupos basándose en el aspecto general de la planta (*Cistus* y *Ledon*). Tournefort apostó por la proposición de dos géneros (*Cistus* y *Helianthemum*) que, posteriormente, Linneo reunió en uno sólo (*Cistus*, excepto las especies americanas conocidas que incluyó en los géneros *Hudsonia* y *Lechea*).

Pourret fue el primer botánico de la época de Linneo en hacer un ensayo monográfico del género *Cistus* que no sería publicado hasta más de un siglo después por Timbal-Lagrave (Cistographie, 1875). El análisis que Dunal realizó y publicó De Candolle en su "Prodromus" (1824), reunió todos los conocimientos adquiridos hasta la fecha sobre las Cistáceas. Este trabajo fue seguido por la colección, no analítica, de descripciones y dibujos que Sweet realizó (Cistineae, 1825-1830) sobre 33 especies de la familia Cistaceae (14 especies y sus variedades e híbridos). La taxonomía del género *Cistus* ha estado basada tradicionalmente en caracteres vegetativos (forma de la hoja, número de nervios, pelosidad) y reproductivos (número de sépalos, color de pétalos, longitud del estilo y número de valvas de los frutos). La diversidad de clasificaciones del género (Dunal 1824; Spach 1836; Willkomm 1856; Grosser 1903; Dansereau 1939; Demoly & Montserrat 1993) refleja cierta desorientación producida por la existencia de formas híbridas. Timbal-Lagrave (1861) reconoció el problema fundamental del género: la hibridación; pero es Bornet quién, con sus experimentos publicados posteriormente por M. Gard (1910; 1912; 1914), demostró que la hibridación se produce comúnmente entre un gran número de especies, así como que la descendencia es inviable en unos casos pero no en otros.

Es en el siglo XX cuando aparecen obras fundamentales en el estudio de las Cistáceas, como son la obra de Willkomm (Cistinearum orbis veteris descriptio monographica, 1856), la publicación de Grosser (Cistaceae, 1903), la monografía de Dansereau (Monographie du genre *Cistus*, 1939) y las monografías de Martín Bolaños y Guinea (Jarales y jaras, 1949; Cistáceas españolas, 1954).

4.2. Descripción del género

El género *Cistus* comprende 21 especies, 16 de las cuales se encuentran distribuidas a lo largo de la cuenca del Mediterráneo y 5 son endémicas de las Islas Canarias (Apéndice 1). Dentro del género se reconocen tres subgéneros (Dansereau 1939; Demoly & Montserrat 1993):

- Subgénero *Cistus* L., que engloba 10 especies cuyas flores presentan 5 sépalos, pétalos color rosa-púrpura, estilo de tamaño similar o superior a la longitud de los estambres (a excepción de *C. parviflorus* que presenta un estilo sésil), polen con exina rugulosa de 1,4 µm de grosor (Saenz 1979), y placenta polisperma. De las 10 especies, cinco se encuentran presentes en la cuenca del Mediterráneo (*Cistus albidus* L., *C. creticus* L., *C. crispus* L., *C. heterophyllus* Desf., *C. parviflorus* Lam.) mientras que las otras cinco son endémicas de las islas Canarias (*Cistus chinamadensis* Bañares et Romero, *C. horrens* Demoly, *C. ochreatus* C. Sm. ex Buch, *C. osbeckiifolius* Webb ex Christ y *C. symphytifolius* Lam.).

- Subgénero *Leucocistus* Willk., formado por ocho especies (*C. ladanifer* L., *C. laurifolius* L., *C. monspeliensis* L., *C. populifolius* L., *C. pouzolzii* Delile, *C. psilosepalus* Sweet, *C. salviifolius* L. y *C. albanicus* E.F. Warb. ex Heywood) que presentan flores con 3 o 5 sépalos, pétalos de color blanco, estilo sésil, subsésil o de longitud inferior a la de los estambres, polen con exina reticulada o retipilada de 4,2 µm de grosor (Saenz 1979), y placenta polisperma.

- Subgénero *Halimioides* (Willk.) Demoly & P. Monts. Las tres especies (*C. clusii* Dunal, *C. libanotis* L., y *C. munbyi* Pomel) contenidas en este subgénero presentan flores con 3 sépalos, pétalos de color blanco, estilo inferior en longitud a los estambres, polen con exina estriada y de 2,8 µm de grosor (Saenz 1979), y placenta oligosperma.

Todas las especies de *Cistus* (Fig. 4 y 5) (Martín Bolaños & Guinea 1949; Demoly & Montserrat 1993) son arbustos con hojas opuestas, enteras, pecioladas y/o sentadas (*Cistus heterophyllus* presenta hojas basales pecioladas y hojas superiores sentadas), y desprovistas de estípulas. Además las especies de *Cistus* (Gülz *et al.* 1996) presentan, en

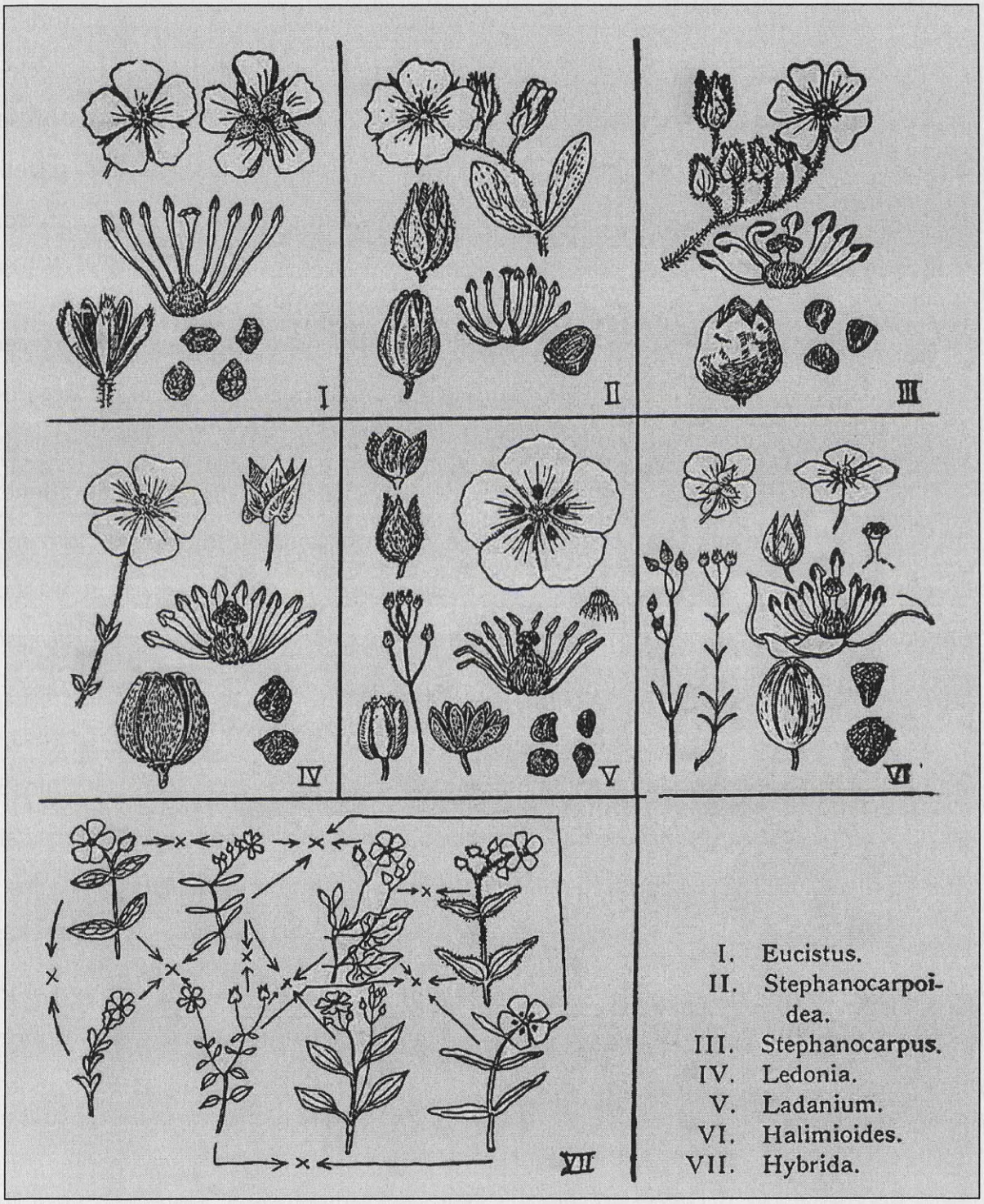


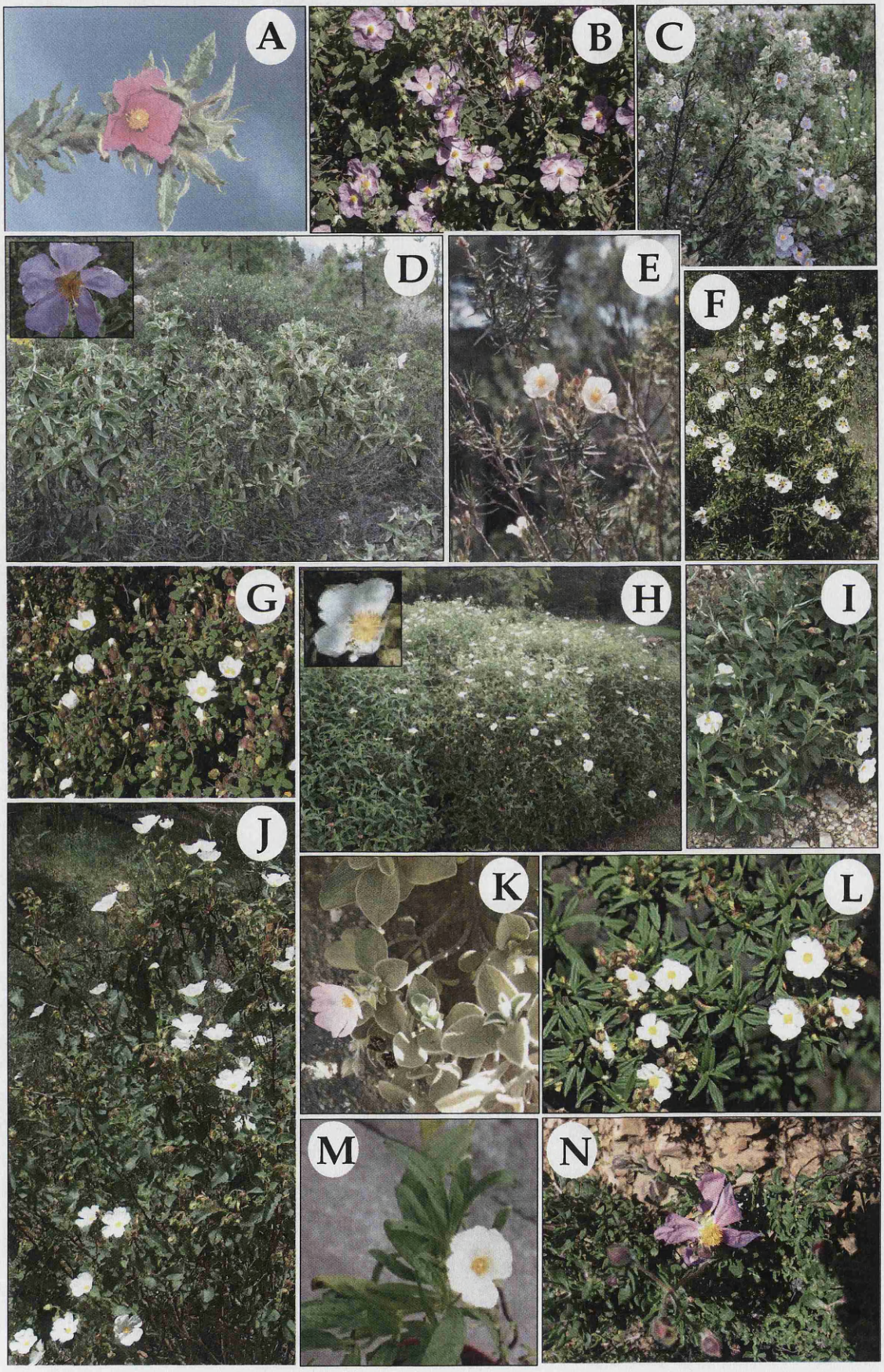
Fig. 4. Representación gráfica de los caracteres diagnóstico de las especies ibéricas incluidas en el género *Cistus* (Martín Bolaños & Guinea 1949).

las hojas y tallos, glándulas (pediceladas o sentadas) que almacenan y sintetizan un exudado resinoso (llamado ládano) formado por compuestos derivados del terpeno (Pascual Teresa *et al.* 1986 y referencias incluidas). Este exudado es muy abundante en la especie *C. ladanifer* siendo esta la razón por la cual comúnmente a esta especie se la conoce como jara pringosa. A excepción de *C. populifolius*, todas las especies presentan distintos tipos de pelos: unicelulares o pluricelulares, simples o fasciculados, estrellados con un número variable de ramas (4-20), glandulíferos, peltados o escuamiformes localizados en distintas zonas de la planta (hojas, tallo, sépalos, fruto, Gülz *et al.* 1996).

Las flores, solitarias (*C. ladanifer* y *C. salviifolius*) o reunidas en inflorescencias (corimbo, *C. albidus*; umbela, *C. laurifolius*; capítulo, *C. crispus*; cima escorpioide, *C. monspeliensis*) presentan tres o cinco sépalos según la especie. La formación de los sépalos es variable en el género, aunque dentro de una misma especie se mantienen. Se observan sépalos ovados, ovado-lanceolados, ovado-acuminados, acorazonados, acuminados u ovalados. Los pétalos (5) son de color rosa-púrpura (representantes del subgénero *Cistus*) o de color blanco (representantes de los subgéneros *Leucocistus* y *Halimioides*). En ambos casos los pétalos presentan una mácula amarilla en la base y sólo en *C. ladanifer* var. *maculatus* aparece además una mancha púrpura intensa. Los estambres se presentan en número alto y variable entre las especies (por ejemplo, 50, *C. pouzolzii*; 100-120, *C. populifolius*; más de 200, *C. ochreatus*), son todos fértiles y se sitúan alrededor del ovario. El estilo tiene una longitud diferente entre las especies (por ejemplo, sésil en *C. ladanifer*; de longitud inferior a la de los estambres en *C. monspeliensis*; de longitud igual a la de los estambres en *C. albidus*; de longitud superior a la de los estambres en *C. osbeckiifolius*), teniendo este carácter una gran importancia taxonómica. El ovario, formado por 5 carpelos (excepto en *C. ladanifer* donde está

→

Fig. 5. Representantes del género *Cistus*. A, *C. crispus* (P. Vargas); B, *C. creticus* (B. Guzmán); C, *C. albidus* (B. Guzmán); D, *C. horrens* (B. Guzmán); E, *C. clusii* (B. Guzmán); F, *C. ladanifer* subespecie *ladanifer* (B. Guzmán); G, *C. salviifolius* (B. Guzmán); H, *C. psilosepalus* (B. Guzmán); I, *C. laurifolius* (B. Guzmán); J, *C. populifolius* (B. Guzmán); K, *C. parviflorus* (P. Vargas); L, *C. monspeliensis* (http://www.gobcan.es); M, *C. albanicus* (Robert G. Page); N, *C. heterophyllus* (B. Guzmán).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formado por 6-12 carpelos) da lugar a un fruto en forma de cápsula que se abre mediante dehiscencia loculicida en 5 valvas (excepto C. *ladanifer* que desarrolla frutos con 6-12 valvas).

Referencias bibliográficas

Ahlberg PE, Clark JA, Luksevics E (1996) Rapid braincase evolution between *Panderichthys* and the earliest tetrapods. *Nature* **381**, 61-64.

Angenent GC, Colombo L (1996) Molecular control of ovule development. *Trends in Plant Science* **1**, 228-232.

Arrington JM, Kubitzki K (2003) Cistaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 62-70. Springer, Berlin.

Bosch J (1992) Floral biology and pollinators of three co-occurring *Cistus* species (Cistaceae). *Botanical Journal of the Linnean Society* **109**, 39-55.

Brandt U, Gottsberger G (1988) Flower phenology. Pollinating insects and breeding systems in *Cistus, Halimium* and *Tuberaria* species in Portugal. *Lagascalia* **15**, 625-634.

Calderón G (1992) Cistaceae. *Flora del Bajío y de regiones adyacentes* **2**, 1-27.

Carroll RL (2000) Towards a new evolutionary synthesis. *Trends in Ecology & Evolution* **15**, 27-32.

Carroll SB (2001) The big picture. *Nature* **409**, 669.

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. *Nature* **353**, 31-37.

Crews D (1994) Temperature, steroids and sex determination. *Journal of Endocrinology* **142**, 1-8.

Cubas P, Vincent C, Coen E (1999) An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157-161.

Chase MW, et al. (1993) Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene rbcL. *Annals of the Missouri Botanical Garden* **80**, 528-548+550-580.

Dansereau P (1939) Monographie du genre *Cistus*. *Boissiera* **4**, 1-90.

Darwin C (1859) *The Origin of Species by Means of Natural Selection*, London.

Das OP, Messing J (1994) Variegated phenotype and developmental methylation changes of a maize allele originating from epimutation. *Genetics* **136**, 1121-1141.

Demoly JP (1996) Les hybrides binaires rares du genre *Cistus* L. (*Cistaceae*). *Anales Jardin Botanico de Madrid* **54**, 241-254.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Doebley J, Lukens L (1998) Transcriptional regulators and the evolution of plant form. *Plant Cell* **10**, 1075-1082.

Dunal (1824) Cistineae in De Candolle. A. P. *Prodromus Systematis Naturalis Regni Vegetabilis* **I**, 263-286.

Erwin DH (2000) Macroevolution is more than repeated rounds of microevolution. *Evolution & Development* **2**, 78-84.

Fernald ML (1917) *Helianthemum dumosum* on the mainland of New England. *Rhodora* **19**, 58-60.

Ford VS, Gottlieb LD (1992) *Bicalyx* is a natural homeotic floral variant. *Nature* **358**, 671-673.

Gard M (1910) Recherches sur les hybrides artificiels des Cistes, obtenus par M. Ed. Bornet. Premier mémoire: Notes inédites et résultats expérimentaux. *Annales des Sciences Naturelles, Botanique* **9**, 71-115.

Gard M (1912) Recherches sur les hybrides artificiels de Cistes, obtenus par M. Ed. Bornet. Deuxième mémoire: Les espèces et les hybrides binaires. *Beihefte zum Botanischen Centralblatt* **2**, 306-394.

Gard M (1914) Recherches sur les hybrides artificiels de Cistes, obtenus par M. Ed. Bornet. Troisième mémoire: Les hybrides dérivés et les hybrides complexes. *Beihefte zum Botanischen Centralblatt* **2**, 306-394.

Gaston KJ, Chown SL (1999) Geographic range size and speciation. In: *Evolution of biological diversity* (eds. Magurran AE, May D), p. 329. Oxford University Press, Oxford.

Gould SJ (1977) *Ontogeny and phylogeny* Harvard University Press, Cambridge.

Grantham T (2007) Is macroevolution more than successive rounds of microevolution? *Palaeontology* **50**, 75-85.

Grosser W (1903) Cistaceae. In: *Dans Pflanzenreich* (ed. Engler A), p. 161, Berlin.

Guinea E (1954) *Cistáceas españolas (con exclusión del género Cistus)* Instituto Forestal de Investigaciones y Experiencias, Madrid.

Gülz PG, Herrmann T, Hangst K (1996) Leaf trichomes in the genus *Cistus*. *Flora* **191**, 85-104.

Hall BK (1999) *Evolutionary developmental biology* Kluwer Academic, Dordrecht.

Hall BK, Olson WM (2003) *Keywords & concepts in Evolutionary Developmental Biology* Harvard University Press, Cambridge.

Herrera J (1987) Flower and fruit biology in southern spanish Mediterranean shrublands. *Annals of the Missouri Botanical Garden* **74**, 69-78.

Hileman LC, Kramer EM, Baum DA (2003) Differential regulation of symmetry genes and the evolution of floral morphologies. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12814-12819.

Hintz M, Bartholmes C, Nutt P, *et al.* (2006) Catching a "hopeful monster": shepherd's purse (*Capsella bursa-pastoris*) as a model system to study the evolution of flower development. *Journal of Experimental Botany* **3**, 1-12.

Irish VF (2003) The evolution of floral homeotic gene function. *BioEssays* **25**, 637-646.

Jablonka E, Lamb MJ (1998) Epigenetic inheritance in evolution. *Journal of Evolutionary Biology* **11**, 159-183.

Jablonski D (1987) Heritability at the species level: analysis of geographic ranges of Cretaceous molluscs. *Science* **238**, 360-363.

Jablonski D (2005) Mass extinctions and macroevolution. *Paleobiology* **31**, 192-210.

Jablonski D (2007) Scale and hierarchy in macroevolution. *Paleontology* **50**, 87-109.

Jablonski D, Hunt E (2006) Larval ecology, geographic range, and species survivorship in Cretaceous molluscs. Organismal vs. species-level explanations. *American Naturalist* **168**, 556-564.

Jablonski D, Roy K (2003) Geographic range and speciation in fossil and living molluscs. *Proceedings of the Royal Society of London, B* **270**, 401-406.

Jackson JBC (1995) Constancy and change of life in the sea. In: *Extinction Rates* (eds. Lawton JH, May RM), pp. 45-54. Oxford University Press, Oxford.

Jackson JBC, Cheetham AH (1999) Tempo and mode of speciation in the sea. *Trends in Ecology & Evolution* **14**, 72-77.

Jones KE, Purvis A, Gittleman JL (2003) Biological correlates of extinction risk in bats. *American Naturalist* **161**, 601-614.

Kendrick P, Crane PR (1997) *The Origin and Early Diversification of Land Plants* Smithsonian Institution Press, Washington, DC.

Kutschera U, Niklas KJ (2004) The modern theory of biological evolution: an expanded synthesis. *Dier Naturwissenschaften* **91**, 255-276.

Linnaeus C (1753) *Species Plantarum*, Estocolmo.

Martín Bolaños M, Guinea E (1949) *Jarales y Jaras (Cistografia Hispanica)* Ministerio de Agricultura. Direccion General de Montes, Caza y Pesca Fluvial. Instituto Forestal de Investigaciones y Experiencias, Madrid.

Meyer P, Linn F, Heidmann I, *et al.* (1992) Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. *Molecular and General Genetics* **234**, 345-352.

Newman SA, Müller GB (2000) Epigenetic mechanisms of character origination. *Journal of Experimental Zoology. Part B, Molecular and developmental evolution.* **288**, 304-317.

Pascual Teresa J, de, Urones JG, Marcos IS, Basabe P, Garrido NM (1986) Diterpenoid and other components of *Cistus laurifolius*. *Phytochemistry* **25**, 1185-1187.

Pelaz S, Ditta GS, Baumann E, Wisman E, Yanofsky MF (2000) B and C floral organ identity functions requiere SEPALLATA MADS-box genes. *Nature* **405**, 200-203.

Pigliucci M (2001) *Phenotypic plasticity: beyond nature and nurture* The Johns Hopkins University Press, Baltimore, Maryland.

Ponzo A (1921) Considerazioni sulle Cistaceae. *Nuovo Giorno Botanoco Italia* **28**, 157-173.

Purvis A, Jones KE, Mace GM (2000) Extinction. *BioEssays* **22**, 1123-1133.

Rubidge BS, Sidor CA (2001) Evolutionary patterns among Permo-Triassic therapsids. *Annual Review of Ecology & Systematics* **32**, 449-480.

Saenz C (1979) Pollen morphology of Spanish Cistaceae. *Grana* **18**, 91-98.

Sage RF, Monson RK (1999) *C_4 plant biology* Academic Press, London.

Sandvik H (2000) A New Evolutionary Synthesis: Do We Need One?" Trends inSandvik, H. (2000) "A New Evolutionary Synthesis: Do We Need One? *Trends in Ecology and Evolution* **15**, 205.

Sarkar S (1998) *Genetics and reductionism* Cambridge University Press, Cambridge.

Savolainen V, Chase MW, Hoot SB, *et al.* (2000) Phylogenetics of flowering plants based on combined analysis of plastid *atpB* and *rbc*L gene sequences. *Systematic Biology* **49**, 306-362.

Schlichting CD, Smith H (2002) Phenotypic plasticity: linking molecular mechanisms with evolutionary outcomes. *Evolutionary Ecology* **16**, 189-211.

Shine R (1995) A new hypothesis for the evolution of viviparity in reptiles. *American Naturalist* **145**, 809-823.

Simons AM (2002) The continuity of microevolution and macroevolution. *Journal of Evolutionary Biology* **15**, 688-701.

Spach E (1836) Conspectus monographiae Cistacearum. *Annales des Sciences Naturelles, Botanique* **6**, 357-375.

Stearns SC, Hoekstra RF (2001) *Evolution: an introduction* Oxford University Press, New York.

Sweet R (1825-1830) *Cistineae. The natural order of Cistus, or Rock-Rose.*

Talavera S, Bastida F, Ortiz PL, Arista M (2001) Pollinator attendance and reproductive success in *Cistus libanotis* L. (Cistaceae). *International Journal of Plant Sciences* **162**, 343-352.

Talavera S, Gibbs PE, Herrera J (1993) Reproductive biology of *Cistus ladanifer* (Cistaceae). *Plant Systematics and Evolution* **186**, 123-134.

Theissen G, Becker A, Di Rosa A, *et al.* (2000) A short history of MADS-box genes in plants. *Plant Molecular Biology* **42**, 115-149.

Theissen G, Saedler H (1995) MADS-box genes in plant ontogeny and phylogeny: Haeckel's "biogenetic law" revisited. *Current Opinion in Genetics & Development* **5**.

Timbal-Lagrave PÉ (1875) Reliquiae Pourretianae ("Cistographie"). *Bulletin de la Societé des Sciences Physiques et Naturelles de Toulouse* **2**, 1-147.

Timbal-Lagrave PMÉ (1861) Etudes sur quelques Cistes de Narbonne *Mémoires de l'Académie des Sciences de Toulouse* **5**, 28-48.

Tournefort JP (1718) *A voyage into the Levant*, London.

Valentine JW, Jablonski D, Erwin DH (1999) Fossils, molecules and embyos: new perspectives on the Cambrian explosion. *Development* **126**, 851-859.

Vermeji GJ (1987) *Evolution and Escalation* Princeton University Press, Princeton.

Vermeji GJ (1994) The evolutionary interaction among species: selection, escalation and coevolution. *Annual Review of Ecology & Systematics* **25**, 219-236.

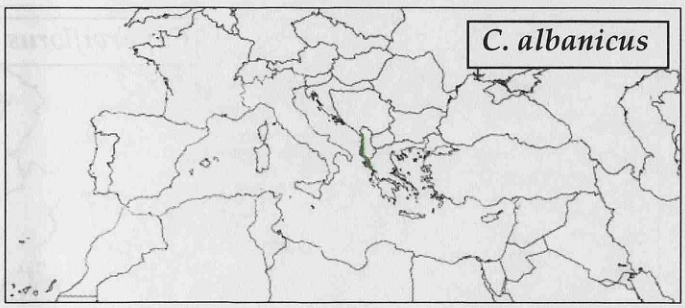
Willkomm M (1856) *Cistinearum orbis veteris descriptio monographica. Icones et Descriptiones Plantarum.*

Zhou Z, Zheng S (2003) The missing link in Ginkgo evolution. *Nature* **423**, 821-822.

Zimmer C (1998) *At the water's edge: macroevolution and the transformation of life* Free Press, New York.

Appendix 1

Distribution maps of *Cistus* species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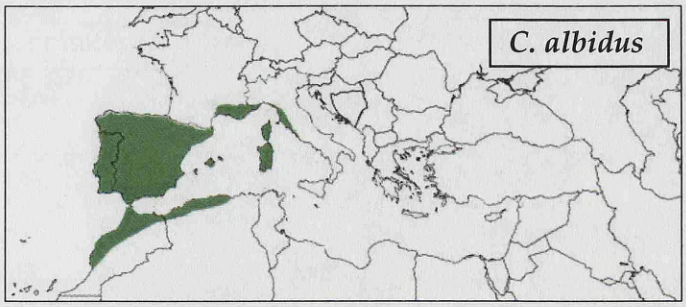
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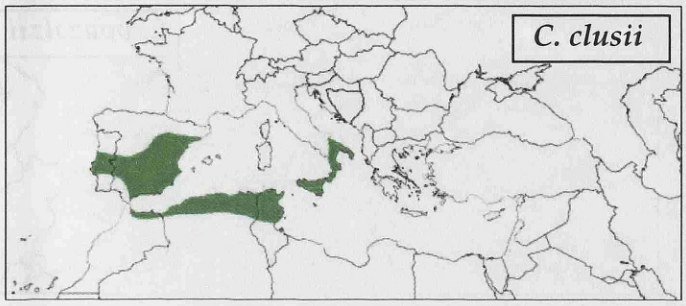
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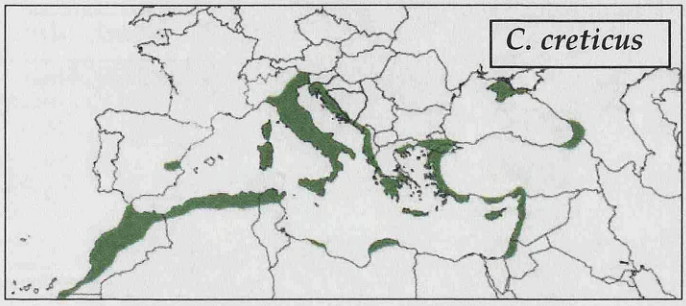
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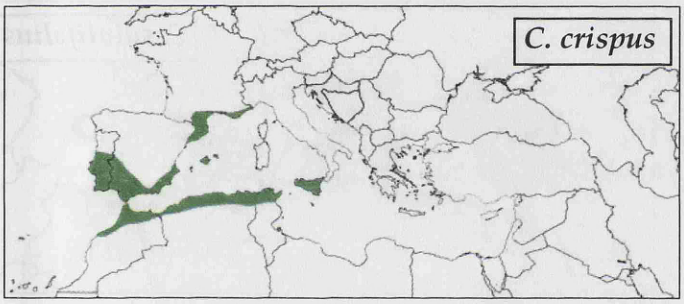
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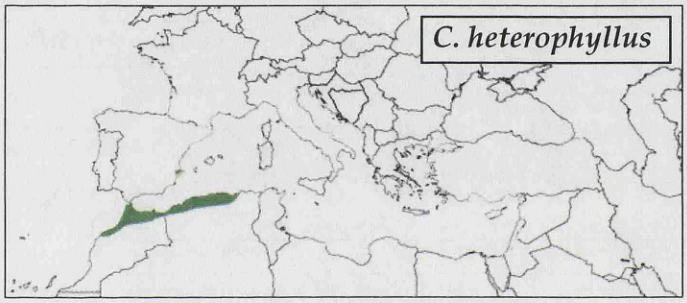
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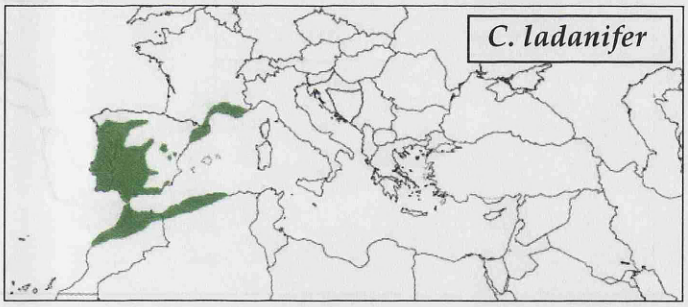
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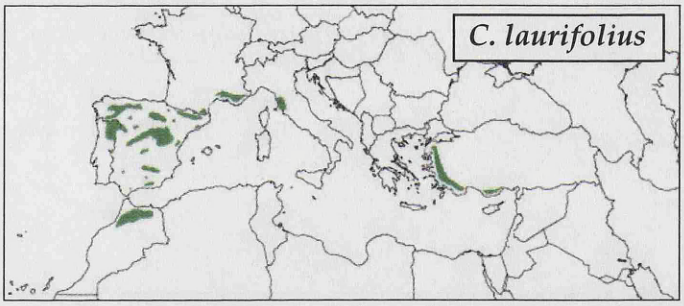
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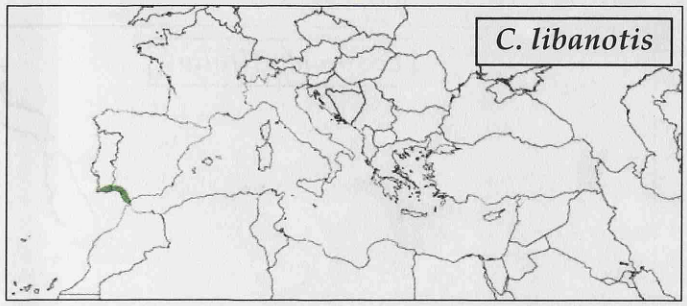
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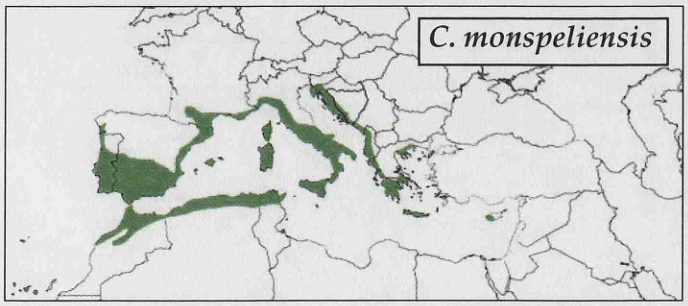
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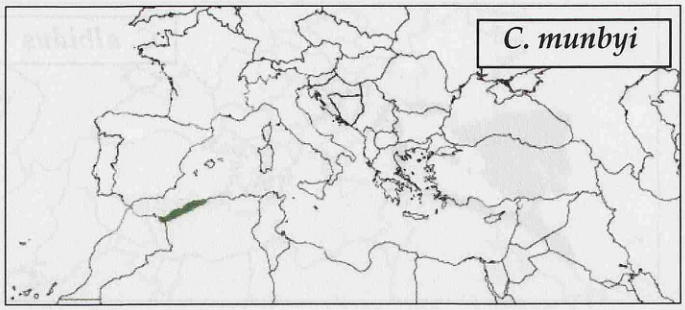
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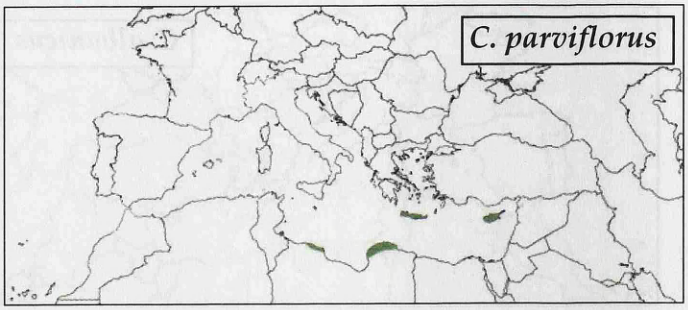
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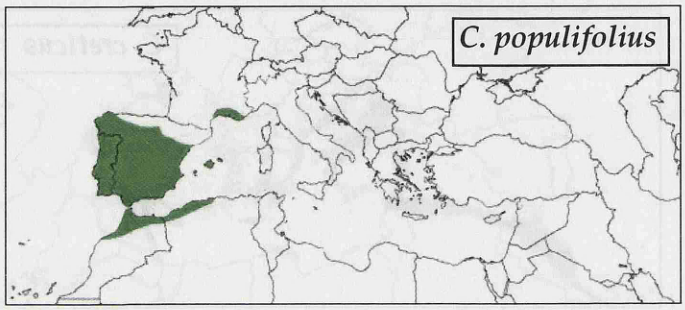
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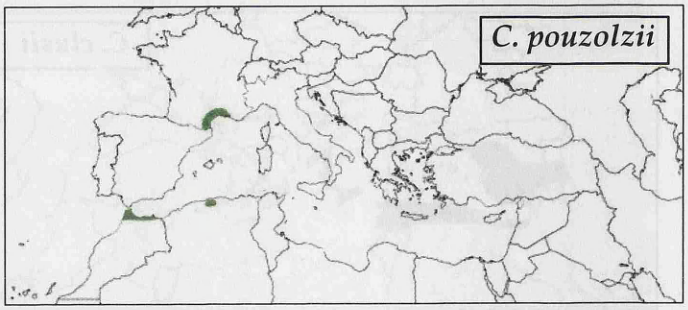
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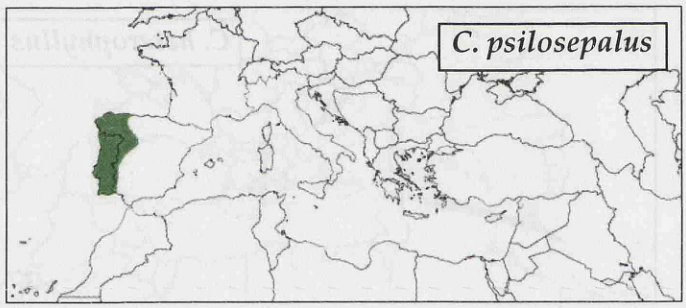
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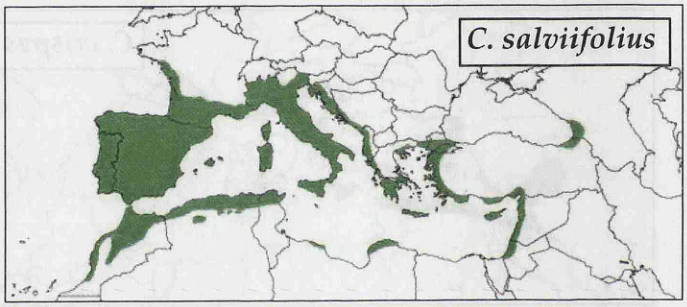
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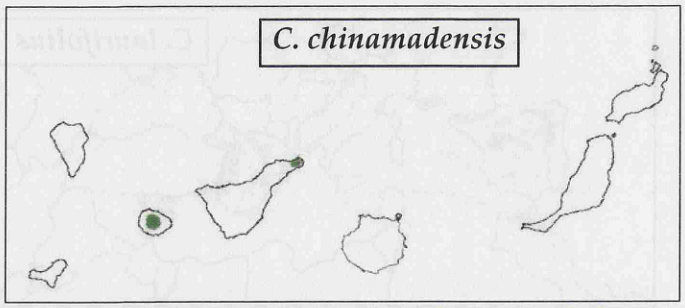
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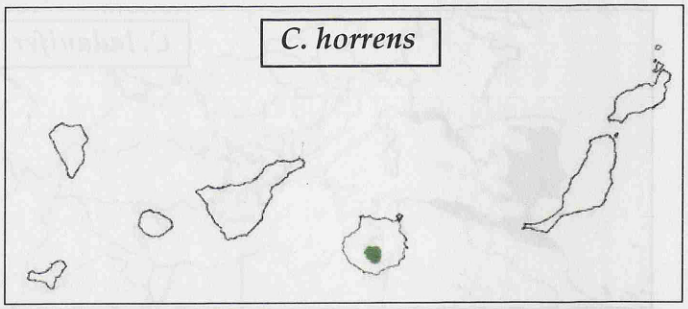
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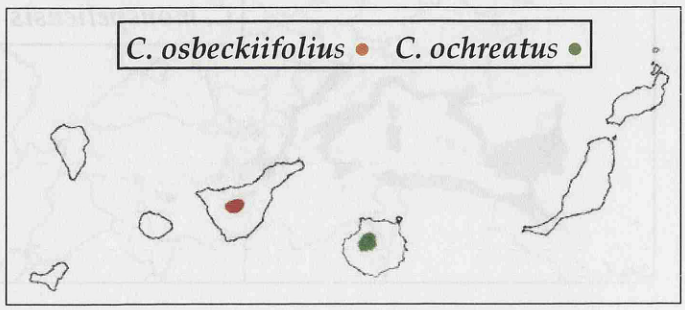
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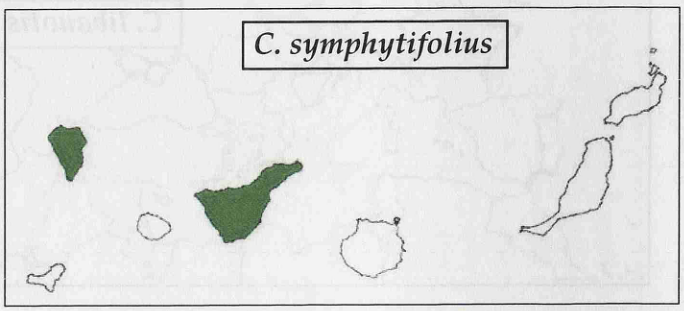
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2

Phylogenetic insights into the Cistaceae (Malvales) based on plastid *rbcL* and *trnL-trnF* sequences

Abstract

The Cistaceae consists of eight genera and about 180 species, and some taxonomic limits within the family remains unresolved when using exclusively morphological data. In the present study a phylogenetic analysis of 46 species, representing the different groups of the Cistaceae, is inferred using coding (*rbcL*) and spacer (*trnL-trnF*) regions of plastid DNA. The firm set of morphological synapomorphies that indicates the monophyly of the family is supported by both Bayesian and parsimony analyses. Four major lineages can be distinguished within the Cistaceae: (1) a stem-based clade containing *Fumana* species; (2) the *Helianthemum* s.l. clade, containing two sister groups, one of species from the New World (*Crocanthemum, Hudsonia*) and other with species from the Old World (*Helianthemum* s. str.); (3) the *Tuberaria* clade; (4) a cohesive complex consisting of *Halimium* and *Cistus* species. Evolutionary shifts of 14 key characters in the Cistaceae are analysed on the most plausible phylogenetic hypothesis. Reconstruction of character evolution (ovule position) reaffirms the ancestral condition of anatropous ovules of *Fumana* within the Cistaceae, which was independently interpreted by Nandi comparing Malvales floral development traits. Genera distribution optimization reveals an early divergence of the Mediterranean-European genera related to tropical vegetation, as complemented by paleobotanical data. The naturalness of the *Cistus-Halimium* assemblage is further supported by a cytological synapomorphy (gametophytic chromosome number of n=9).

Key words: Centre of diversification, character reconstruction, Malvales, *rbcL*, Systematics, *trnL-trnF*

1. Introduction

No consensus for a particular placement of the Cistaceae within the angiosperm has been observed in the last decades (Bixales, Takhtajan 1987; Violales, Cronquist 1988; Malvales, Dahlgren 1989; Violales, Thorne 1992; Cistales, Takhtajan 1997). The families of the Malvales, including the Cistaceae, share several vegetative and seed characters (Alverson *et al.* 1998; Nandi 1998a, 1998b; Kubitzki & Chase 2003; Horn 2004), but synapomorphies are difficult to find for this taxonomic order. The erratic pattern of features distribution across families and the lack of morphological studies in some families have made necessary the use of molecular techniques to propose order circumscription. In this context, molecular analyses based on plastid and nuclear sequences have confirmed the inclusion of the Cistaceae within Malvales, forming the Dipterocarpalean clade together with two primarily tropical families (Dipterocarpaceae, Sarcolaenaceae) (Savolainen *et al.* 2000; Soltis *et al.* 2000). Close relationships between these three families were earlier stressed by Nandi (1998b) as supported by the presence of the peculiar bixoid chalazal region of the seed coat (character previously found in Cistaceae, Bixaceae and Cochlospermaceae seeds by Corner (1976) and Takhtajan (1992)) and wood anatomical similarities between Dipterocarpaceae (subfam. Monotoideae) and Cistaceae (Baas & Werker 1981).

The Cistaceae is a medium-size family (eight genera, 180 species) typically consisting of heliophyte shrubs, subshrubs and herbs occurring in open areas on poor soils. Distributed in temperate and subtropical regions of the northern hemisphere, the Cistaceae shows the highest generic and species diversity in the Mediterranean floristic region. In fact, five (*Cistus, Fumana, Halimium, Helianthemum, Tuberaria*) of the eight genera are native to this region while the other three (*Crocanthemum, Hudsonia, Lechea*) inhabit temperate regions of the New World. The eight genera share hermaphrodite, actinomorphic, hypoginous flowers with three or five sepals (usually outer smaller than inner ones) opposite to petals (when petals are present). Cleistogamous flowers can be commonly found in particular genera (*Lechea, Helianthemum, Crocanthemum*). The androecium has numerous fertile stamens (except the outer, sterile stamens of *Fumana*)

and the gynoecium is formed by an ovary three- or five-carpelleted in *Cistus* (although *C. ladanifer* displays 6-12 carpels) and a solitary style with a single capitate or discoid stigma (except for the three free stigmas of *Lechea*) (Table 1).

The taxonomy of the Cistaceae has been based primarily on vegetative (growth form, leaf arrangement and attachment) and reproductive (sepal number, petal number and color, style length, stamen fertility, number of fruit valves) characters (Table 1). Several taxonomic treatments are available for the family since the eightieth century, which propose different generic and infrageneric classifications (Linnaeus 1753; Dunal 1824; Spach 1836; Willkomm 1856; Ponzo 1921; Martín Bolaños & Guinea 1949) (Table 2). Eight genera were recognised using morphological and molecular characters in the last taxonomic assessment (Arrington & Kubitzki 2003). Particular points of disagreement with the previous classifications of the Cistaceae are whether recognition of three genera (*Crocanthemum, Cistus, Halimium*) as independent taxonomic entities. The circumscription of the New World species of *Crocanthemum* within the family is one of the most problematic, with arguments favouring their placement either in this genus (Martín Bolaños & Guinea 1949; Arrington & Kubitzki 2003) or in *Helianthemum* (Fernald 1917; Calderón 1992). On the other hand, the species of *Cistus* and two studied species of *Halimium* form a cohesive natural group (Guzmán & Vargas 2005) and share some morphological and karyological characters, result requiring an in-depth study including all *Halimium* species.

A phylogenetic analysis of the Cistaceae has not been published to date. In the present study, we utilize DNA sequence data from the plastid *rbcL* gene and *trnL-trnF* spacer to: (1) test the monophyly of the Cistaceae; (2) identify monophyletic groups in the Cistaceae to be contrasted with previous generic circumscriptions; (3) infer sister-group relationships within the Cistaceae; and (4) offer new insights into the evolutionary change in the Cistaceae by reconstructing key characters.

Table 1. Morphological characters and states on which the taxonomy of the Cistaceae genera has been mostly based on. Data were taken from Martín Bolaños & Guinea (1949), Calderón (1992), Demoly & Montserrat (1993), Ukraintseva (1993), Nandi (1998a; 1998b), Arrington & Kubitzki (2003), and own observations

	No. of sepals	No. of petals	Petal color	Staminodes	No. of stamens	No. of stigmas	Style length
Cistus L.	3-5	5	White, purple	No	Many (50-200)	1	Sessile, short, elongate
Crocanthemum Spach	5	5	Yellow	No	Few-many	1	Short
Fumana (Dunal) Spach	5	5	Yellow	Yes	Numerous (26-40)	1	Elongate
Halimium (Dunal) Spach	3(5)	5	White, yellow	No	Numerous-many (20-100	1	Sessile, short
Helianthemum Mill.	5	5	White, yellow, purple	No	Few-many (7- 100)	1	Elongate
Hudsonia L.	5	5	Yellow	No	Few-numerous (10-30)	1	Elongate
Lechea L.	5	3	Dark red	No	Few-numerous (3-25)	3	Sessile
Tuberaria (Dunal) Spach	5	5	Yellow	No	Few-numerous (10-50)	1	Sessile, short

	No. of carpels	Ovule position	Embryo shape	Pollen-type	Flower type
Cistus L.	5-12	Orthotropous	Circinate	Cistus	Chasmogamous
Crocanthemum Spach	(2)3	Orthotropous	Curved	Crocanthemum	Chasmogamous, cleistogamous
Fumana (Dunal) Spach	3	Anatropous	Curved	Fumana, Helianthemum	Chasmogamous
Halimium (Dunal) Spach	3	Orthotropous	Curved-circinate	Cistus	Chasmogamous
Helianthemum Mill.	3	Orthotropous	Simple plicate, biplicate	Helianthemum	Chasmogamous, cleistogamous
Hudsonia L.	3	Orthotropous	Curved	Hudsonia	Chasmogamous
Lechea L.	3	Orthotropous	Linear, slightly curved	Lechea	Chasmogamous, cleistogamous
Tuberaria (Dunal) Spach	3	Orthotropous	Triangular	Cistus	Chasmogamous

	Leaf attachment	Leaf arrangement[1]	Gametophytic number of chromosomes (n =)	Life form
Cistus L.	Exstipulate	opposite	9	Shrubs
Crocanthemum Spach	Exstipulate	alternate	10	Shrubs
Fumana (Dunal) Spach	Exstipulate, stipulate	alternate, opposite	16	Dwarf shrubs
Halimium (Dunal) Spach	Exstipulate	opposite	9	Shrubs, suffruticoses
Helianthemum Mill.	Exstipulate, stipulate	opposite	10(5, 12, 20)-11	Shrubs, subshrubs, herbs
Hudsonia L.	Exstipulate	alternate	10	Low shrubs
Lechea L.	Exstipulate	alternate	-	Perennial suffruticoses
Tuberaria (Dunal) Spach	Exstipulate, stipulate	opposite, alternate	12, 18, 24	Annual or perennial herbs

[1] On vegetative branches

Table 2. Taxonomic treatments of the Cistaceae across the history

Linnaeus (1753-1756)	Dunal (1824)	Spach (1836)	Willkomm (1856)
Genus *Cistus* L.	I. Genus *Cistus* Tourn.	Tribus I. *Cisteae* Spach	Subfamil. I. Cistoideae Willk.
	Sect. I. *Erythrocistus* Dunal	Sect. I. *Fumanieae* Spach	Tribus I. Normales Willk.
Genus *Hudsonia* L.	Sect. II. *Ledonia* Dunal	Genus *Fumana* Dunal	Subtribus 1. Cisteae Willk.
		Sect. II. *Cistineae* Spach	Genus *Cistus* Tourn.
Genus *Lechea* L.	II. Genus *Helianthemum* Tourn.	Subdivisio 1. *Helianthemoideae* Spach	Subgenus I. *Erythrocistus* Dunal
	Sect. I. *Halimium* Dunal	Genus *Helianthemum* (Tourn.) Spach	Sect. 1. *Macrostylia* Willk.
	Sect. II. *Lecheoides* Dunal	Sect. I. *Aphananthemum* Spach	Sect. 2. *Brachystylia* Willk.
	Sect. III. *Tuberaria* Dunal	Sect. II. *Eriocarpum* Dunal	Sect. 3. *Astylia* Willk.
	Sect. IV. *Macularia* Dunal	Sect. III. *Euhelianthemum* Dunal	Subgenus II. *Leucocistus* Willk.
	Sect. V. *Brachypetalum* Dunal	Sect. IV. *Argyrolepis* Spach	Sect. 4. *Stephanocarpus* Spach
	Sect. VI. *Eriocarpum* Dunal	Genus *Rhodax* Spach	Sect. 5. *Ledonia* Spach
	Sect. VII. *Fumana* Dunal	Genus *Tuberaria* (Dunal)	Sect. 6. *Ladanium* Spach
	Sect. VIII. *Pseudocistus* Dunal	Subdivisio 2. *Cistoideae* Spach	Sect. 7. *Halimioides* Willk.
	Sect. IX. *Euhelianthemum* Dunal	Genus *Halimium* Dunal	Genus *Halimium* Willk.
		Sect. I. *Leucorhodion* Spach	Sect. 1. *Oligospermia* Willk.
	III. Genus *Hudsonia* L.	Sect. II. *Chrysorhodion* Spach	Sect. 2. *Polyspermia* Willk.
		Genus *Ladanium* Spach	Genus *Tuberaria* Spach
	IV. Genus *Lechea* L.	Genus *Rhodocistus* Spach	Sect. 1. *Eutuberaria* Willk.
		Genus *Cistus* (Tourn.) Spach	Sect. 2. *Scorpioides* Willk.
		Sect. I. *Rhodopsis* Spach	Genus *Helianthemum* Willk.
		Sect. II. *Eucistus* Spach	Subgenus I. *Ortholobum* Willk.
		Sect. III. *Ledonella* Spach	Sect. 1. *Brachypetalum* Dunal
		Genus *Stephanocarpus* Spach	Sect. 2. *Eriocarpum* Dunal
		Genus *Ledonia* Spach	Sect. 3. *Euhelianthemum* Dunal
		Subdivisio III. *Heteromerineae* Spach	Sect. 4. *Polystachyum* Willk.
		Genus *Crocanthemum* Spach	Subgenus II. *Plectolobum* Willk.
		Genus *Heteromeris* Spach	Sect. 5. *Chamaecistus* Willk.
		Genus *Taeniostema* Spach	Genus *Crocanthemum* Spach
			Genus *Hudsonia* L.
		Tribus II. *Lechidieae* Spach	
		Genus *Lechea* (L.) Spach	Subtribus 2. Fumaneae Willk.
		Genus *Lechidium* Spach	Genus *Fumana* Spach
			Sect. 1. *Helianthemoides* Willk.
		Genus anomalum	Sect. 2. *Eufumana* Willk.
		Genus *Hudsonia* L.	
			Tribus II. Abnormes Willk.
			Genus *Heteromeris* Spach
			Genus *Taeniostemma* Spach
			Subfamil. Lechidioideae Willk.
			Genus *Lechea* (L.) Spach
			Genus *Lechidium* Spach

Table 2. *(Continued)*

Grosser (1903)	Ponzo (1921)	Demoly and Montserrat (1993) (Iberian species)
Genus *Cistus* L. Group A. Sect. 1. *Rhodocistus* (Spach) Gross. Sect. 2. *Eucistus* Spach Sect. 3. *Ledonella* Spach Group B. Sect. 4. *Stephanocarpus* (Spach) Willk. Sect. 5. *Ledonia* Dunal Group C. Sect. 6. *Ladanium* (Spach) Willk. Sect 7. *Halimioides* Willk. Genus *Halimium* (Dunal) Willk. Group A. Sect. 1. *Spartioides* Gross. Group B. Sect. 2. *Euhalimium* Gross. Sect. 3. *Lecheoides* Dunal Genus *Tuberaria* (Dunal) Spach Sect. 1. *Eutuberaria* Willk. Sect. 2. *Scorpioides* Willk. Genus *Helianthemum* Adans. Subgen. I. *Ortholobum* Willk. Group A. Sect. 1. *Polystachyum* Willk. Sect. 2. *Euhelianthemum* Dunal Sect. 3. *Pseudomacularia* Gross. Group B. Sect. 4. *Eriocarpum* Dunal Sect. 5. *Brachypetalum* Dunal Subgen. II. *Plectolobum* Willk.. Sect. 1. *Chamaecistus* Willk. Sect. 2. *Macularia* Dunal Genus *Fumana* (Dunal) Spach. Genus *Hudsonia* (L.) Genus *Lechea* Kalm. Sect. 1. *Eulechea* Robins Sect. 2. *Lechidium* Torr.	Genus *Cistus* L. Genus *Halimium* (Dunal) Willk. Genus *Heteromeris* Spach Genus *Tuberaria* (Dunal) Spach Genus *Helianthemum* Willk. Genus *Fumana* (Dunal) Spach Genus *Hudsonia* L. Genus *Lechea* L.	Genus *Cistus* L. Subgen. I. *Cistus* L. Subgen. II. *Leucocistus* Willk. Sect. 1. *Ledonia* Dunal Sect. 2. *Ladanium* (Spach) Gren. Subgen. III. *Halimioides* (Willk.) Demoly & P. Monts. Genus *Halimium* (Dunal) Spach Sect. 1. *Halimium* (Dunal) Spach Sect. 2. *Homorhodion* Paiva & I. Nogueira Sect. 3. *Chrysorhodion* Spach Sect. 4. *Commutata* Izco & Jiménez Alb. Genus *Xolantha* Raf. Sect. 1. *Tuberaria* (Dunal) Gallego, Muñoz Garm. & C. Navarro Sect. 2. *Xolantha* Raf. Genus *Helianthemum* Mill. Subgen. I. *Helianthemum* Mill. Sect. 1. *Argyrolepis* Spach Sect. 2. *Lavandulaceum* G. López Sect. 3. *Helianthemum* Mill. Sect. 4. *Brachypetalum* Dunal Sect. 5. *Caput-felis* G. López Subgen. II. *Plectolobum* Willk. Sect. 6. *Pseudocitus* Dunal Sect. 7. *Atlanthemum* (Raynaud) G. López, A. Ortega & Romero García Genus *Fumana* (Dunal) Spach Subgen. I. *Fumana* (Dunal) Spach Subgen. II. *Pomelina* Maire Subgen. III. *Fumanopsis* (Pomel) Janch.

2. Material and Methods

2.1. Sample strategy and DNA sequencing

A total of 53 individuals representing the 21 species of *Cistus*, three of *Crocanthemum*, three of *Fumana*, eight of *Halimium*, eight of *Helianthemum*, one of *Hudsonia*, and two of *Tuberaria* were sampled for *trnL-trnF* and *rbcL* sequencing (Table 3). A particular effort has been done to include representatives of infrageneric taxa (i. e. species representing most of subgenera and sections). Accordingly, we have sequenced one representative of each of the three *Fumana* subgenera (*Fumana, Pomelina, Fumanopsis*), three species of *Crocanthemum* section *Lecheoides* (but none of section *Spartioides* (three species)), one species representing the small genus *Hudsonia* (three species), six species representing three of the five Iberian and one non-Iberian sections of *Helianthemum* subgenus *Helianthemum*, two species representing one of the two sections of *Helianthemum* subgenus *Plectolobum*, one species of each of the two *Tuberaria* sections (*Tuberaria, Xolantha*). Species of *Halimium* and *Cistus* are all included in the present study. Unfortunately we failed in sequencing three species of *Lechea* from herbarium specimens. Total genomic DNA was extracted from material collected in the field, material in the living collections of R. Page, O. Filippi, and the Royal Botanic Garden of Madrid, and from nine herbarium specimens (MA). Field collections were dried in silica gel. DNA was extracted using Kneasy Plant Mini Kit (QIAGEN Inc., California) and amplified using the PCR (Polymerase Chain Reaction) on a Perkin-Elmer PCR System 9700 (California) or an MJ Research (Massachusetts) thermal cycler. Standard primers were used for amplification of the *trnL*(UAA)-*trnF*(GAA) spacer (Taberlet *et al.* 1991), while the *rbcL* exon was amplified in two overlapping segments using the following combination of primers: 1F-724R and 636F-1460R (Savolainen *et al.* 2000). After 1-3 min pretreatment at 94 °C, PCR conditions for *rbcL* and *trnL-trnF* amplification were: 24-28 cycles of 1 min at 94 °C, 1 min at 48-50 °C and 2-4 min at 72 °C. A volume of 1 µL of dimethyl-sulfoxide (DMSO) was included in each 25 µl reaction. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little

Table 3. Cistaceae taxa sequenced for the *rcbL* gene and *trnL-trnF* spacer. Bixaceae, Dipterocarpaceae, Muntingiaceae, Thymelaeaceae, Sarcolaenaceae, Sphaerosepalaceae sequences were obtained from the GenBank. Material source, voucher reference and GenBank accession numbers are also indicated. Taxonomy follows that of Savolainen *et al.* (2000) and Guzmán & Vargas (2005)

Taxon	*Locality/source*	*Voucher*	*trnL-trnF accession no.*	*rbcL accession no.*
Cistus L.				
Cistus albanicus E.F. Warb. ex Heywood	Cultivated	R. G. Page 8cBGA04 (MA)	DQ093030	Forthcoming
Cistus albidus L	Spain, Madrid, Aldea del Fresno	P. Vargas 25PV03 (MA)	DQ093021	Forthcoming
Cistus chinamadensis Bañares et Romero	Canary Islands, La Gomera	Á. Fernández & J. Leralta 44BGA04 (MA)	DQ093033	Forthcoming
Cistus clusii Dunal	Spain, Málaga, Mijas	R. G. Page 8bBGA04 (MA)	DQ093056	Forthcoming
Cistus creticus L.	Greece, Olympus	P. Vargas 209PV04 (MA)	DQ093025	Forthcoming
Cistus crispus L.	Spain, Córdoba, Posadas	B. Guzmán 58BGA04 (MA)	DQ093060	Forthcoming
Cistus heterophyllus Desf.	Morocco, Beni-Hadifa	B. Guzmán 99BGA04 (MA)	DQ093036	Forthcoming
Cistus horrens Demoly	Canary Islands, Gran Canaria, Ayacata	B.Guzmán 2BGA05 (MA)	Forthcoming	Forthcoming
Cistus ladanifer L.	Spain, Madrid, Boadilla del Monte	B. Guzmán 7BGA03 (MA)	DQ093043	Forthcoming
Cistus laurifolius L.	Spain, Jaén, Sierra de Segura	B. Guzmán 13BGA03 (MA)	DQ093052	Forthcoming
Cistus libanotis L.	Spain, Córdoba	R. G. Page 149BGA04 (MA)	DQ093040	Forthcoming
Cistus monspeliensis L.	Portugal, Sagres	B. Guzmán 35BGA04 (MA)	DQ093059	Forthcoming
Cistus munbyi Pomel	Morocco	O. Filippi 4BGA04 (MA)	DQ093053	Forthcoming
Cistus ochreatus C. Sm. ex Buch	Canary Islands, Gran Canaria	R. G. Page 8BGA04 (MA)	DQ093032	Forthcoming
Cistus osbeckiifolius Webb ex Christ	Canary Islands, Tenerife	P. Escobar 48/05 (MA)	Forthcoming	Forthcoming
Cistus parviflorus Lam.	Greece, Crete	O. Filippi 6BGA04 (MA)	DQ093023	Forthcoming
Cistus populifolius L.	Portugal, Ourique	B. Guzmán 20BGA04 (MA)	DQ093049	Forthcoming
Cistus pouzolzii Delile	France	R. G. Page 8tBGA04 (MA)	DQ093054	Forthcoming
Cistus psilosepalus Sweet	Spain, Ávila, Arenas de San Pedro	P. Vargas 7PV03 (MA)	DQ093041	Forthcoming
Cistus salviifolius L.	Spain, Ávila, Arenas de San Pedro	P. Vargas 6PV03 (MA)	DQ093037	Forthcoming
Cistus symphytifolius Lam.	Canary Islands, La Palma, La Cumbrecita	B. Guzmán 143BGA04 (MA)	DQ093057	Forthcoming
Crocanthemum Spach				
Crocanthemum argenteum (S.Watson) Janch.	Mexico, Guanajuato	J. Rzedowski (527770MA)	Forthcoming	Forthcoming
Crocanthemum chihuahuense S.Watson	Mexico, Michoacán	G. Calderón (527771MA)	Forthcoming	Forthcoming
Crocanthemum pringlei (S.Watson) Janch.	Mexico, Guanajuato	G. Calderón (527767MA)	Forthcoming	Forthcoming
Fumana (Dunal) Spach				
Fumana ericoides Pau	Spain, Almería, Cabo de Gata	B.Guzmán 3BGA06 (MA)	Forthcoming	Forthcoming
Fumana fontanesii Clauson ex Pomel	Cultivated	J. Güemes 121BGA04 (MA)	Forthcoming	Forthcoming
Fumana thymifolia (L.) Spach ex Webb	Portugal, Ferrerías	B. Guzmán 53BGA04 (MA)	DQ093015	Forthcoming
Halimium (Dunal) Spach				
Halimium atlanticum Humbert & Maire	Morocco, Tazzeka (1)	RDG14/2006/5	Forthcoming	Forthcoming
Halimium atlanticum Humbert & Maire	Morocco, Bab-Taza (2)	J. Molero *et al.* RDG14/2006/1	Forthcoming	Forthcoming
Halimium atriplicifolium (Lam.) Spach	Spain, Granada, Sierra Almizara (1)	J. M. Martínez 7BGA07 (MA)	Forthcoming	Forthcoming
Halimium atriplicifolium (Lam.) Spach	Spain, Málaga, Coín (2)	R. G. Page 155bBGA05 (MA)	Forthcoming	Forthcoming
Halimium calycinum (L.) K. Koch	Portugal, Cabo Sardao (1)	B. Guzmán 49BGA04 (MA)	DQ093020	Forthcoming
Halimium calycinum (L.) K. Koch	Portugal, Cabo de San Vicente (2)	B. Guzmán 37BGA04 (MA)	Forthcoming	Forthcoming
Halimium halimifolium (L.) Willk. *halimifolium*	Spain, Málaga, Marbella	A. Segura (580185MA)	Forthcoming	Forthcoming
Halimium halimifolium (L.) Willk. *multiflorum* (Salzm. Ex Dunal) Marie	Portugal, Pegoes	E. Monasterio *et al.* (459452MA)	Forthcoming	Forthcoming
Halimium lasianthum (Lam.) Spach *lasianthum*	Spain, Ma, Parque Nacional Alcornocales	P. Vargas 3PV06 (MA)	Forthcoming	Forthcoming

Halimium lasianthum (Lam.) Spach *alyssoides* (Lam.) Greuter	Portugal, Algarve	L. Medina *et al.* (690834MA)	Forthcoming	Forthcoming
Halimium lasiocalicynum (Boiss. & Reut.) Gross ex Engl. *riphaeum* (Pau & Font Quer) Maire	Morocco, Bab-Berred	P. Escobar 665/04 (MA)	Forthcoming	Forthcoming
Halmium ocymoides (Lam.) Willk.	Portugal, Coimbra (1)	R. G. Page 158BGA04 (MA)	Forthcoming	Forthcoming
Halimium ocymoides (Lam.) Willk.	Spain (2)	R. G. Page 158bBGA04 (MA)	Forthcoming	Forthcoming
Halimium umbellatum (L.) Spach *umbellatum*	Spain, Madrid, Tres Cantos	P. Vargas 71BGA04 (MA)	DQ093014	Forthcoming
Halimium umbellatum (L.) Spach *viscosum* (Willk.) O. Bolòs & Vigo	Spain, Ciudad Real, S^a Morena	L. Serra (705587MA)	Forthcoming	Forthcoming
Helianthemum Mill.				
Helianthemum aegyptiacum (L.) Mill.	Spain, Madrid, Rivas VaciaMadrid	P. Vargas 200PV04 (MA)	Forthcoming	Forthcoming
Helianthemum almeriense Pau	Spain, Granada, Calahonda	B. Guzmán 80BGA04 (MA)	Forthcoming	Forthcoming
Helianthemum kahiricum Delile	Morocco	P. Escobar 93/04 (MA)	Forthcoming	Forthcoming
Helianthemum ledifolium (L.) Mill.	Spain, Madrid, Tres Cantos	P. Vargas 185PV05 (MA)	Forthcoming	Forthcoming
Helianthemum marifolium (L.) Mill.	Portugal, Sagres	B. Guzmán 31BGA04 (MA)	Forthcoming	Forthcoming
Helianthemum oelandicum (L.) Dum. Cours.	-	J. M. Martínez 8BGA07 (MA)	Forthcoming	Forthcoming
Helianthemum scopulicolum L.	Cultivated	B. Guzmán 67BGA04 (MA)	DQ093017	Forthcoming
Helianthemum squamatum (L.) Dum. Cours.	Cultivated	B. Guzmán 70BGA04 (MA)	DQ093016	Forthcoming
Hudsonia L.				
Hudsonia tomentosa Nutt.*	USA, MI, dunes N. of Luddington	Chase & Fay 14587	Forthcoming	Forthcoming
Tuberaria Dunal				
Tuberaria guttata (L.) Fourr.	Portugal, Vila do Bispo	B. Guzmán 44BGA04 (MA)	DQ093018	Forthcoming
Tuberaria globulariifolia (Lam.) Gallego	Spain, Orense, Sierra de Xures	J. Martínez 269JM04 (MA)	Forthcoming	Forthcoming
Bixaceae				
Diegodendron Capuron				
Diegodendron humbertii Capuron	Madagascar	Fay *et al.* (1998)	-	Y15138
Dipterocarpaceae				
Anisoptera Korth.				
Anisptera costata Korth.	-	Yuwa-amornpitak,T. *et al.* (unpublished data)	DQ157291	-
Anisoptera marginata Korth.	-	Fay *et al.* (1998)	-	Y15144
Hopea Roxb.				
Hopea hainanensis Merr. & Chun	-	Cho *et al.* (unpublished data)	-	AJ247623.1
Monotes A.D.C.				
Monotes madagascariensis	-	Gamage *et al.* (2006)	AB246543.1	-
Muntingiaceae				
Muntingia L.				
Muntingia calabura L.	-	Fay *et al.* (1998)	-	Y15146
Thymelaeaceae				
Aquilaria Lam.				
Aquilaria beccariana Tiegh.	-	Fay *et al.* (1998)	-	Y15149
Sarcolaenaceae				
Sarcolaena Thouars				
Sarcolaena multiflora Thou.	-	Ducousso *et al.* (2004)	-	AY157715
Sphaerosepalaceae				
Rhopalocarpus Bojer				
Rhopalocarpus sp.	-	Fay *et al.* (1998)	-	Y15148

* Plant material from The Royal Botanic Gardens, Kew, DNA Bank, www.rbgkew.org.uk/data/dnaBank/homepage.html

Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism model 3700 automated sequencer. PCR primers were used for cycle sequencing of the *trnL-F* spacer and the *rbcL* exon. Sequenced data were assembled and edited using the program Seqed (Applied Biosystems, California). The limits of the regions were determined by position of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

2.2. Molecular analysis

DNA sequence variation was used to reconstruct phylogenetic relationships using Bayesian Inference (BI) and Maximum Parsimony (MP). A combination of *rbcL* and *trnL-trnF* data sets of the Cistaceae and eight representatives of families circumscribed to Malvales was performed to investigate monophyly of the Cistaceae and relationships within the family. We chose six of the most closely related families of Malvales as the outgroup: Bixaceae, Dipterocarpaceae, Muntingiaceae, Thymelaeaceae, Sarcolaenaceae, Sphaerosepalaceae (Alverson *et al.* 1998; Soltis *et al.* 2005) (Table 3). Sequences were aligned using Clustal X 1.62b (Thompson *et al.* 1997), with further adjustments by visual inspection. For the Bayesian Inference analysis, the simplest model of sequence evolution that best fits the sequence data was determined using the Hierarchical Likelihood Ratio Test (hLRT) and the Akaike Information Criterion (AIC). Both tests were implemented on each data set using MrModeltest 1.1b (Posada & Crandall 1998; Nylander 2002). The simplest model of evolution found was GTR+G for *trnL-F* and GTR+I+G for *rbcL* data sets. Bayesian Inference analysis was conducted using MrBayes 3.2.1 (Ronquist & Huelsenbeck 2003) with each model substitution fitted to each molecular partition. Two times four chains were run for ten million generations (chain temperature = 0.2; sample frequency = 100). In both runs probabilities converged on the same stable value approximately after generation 1,000,000. A 50% majority–rule consensus tree was calculated using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro *et al.* 2003).

Parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford 1999) with equal weighting of all characters and of transitions/transversions. We performed 1000 random addition replicates of heuristic searches with TBR, holding 100 trees per replicate and the options Multrees (keeping multiple, shortest trees) and Steepepest Descent in effect. Internal support was assessed using 5,000,000 bootstrap replicates (fast stepwise-addition, Mort *et al.* 2000).

Evolutionary patterns of fourteen morphological characters considered to be taxonomically important in the Cistaceae were traced on the Bayesian phylogeny using MacClade 4.06 (Maddison & Maddison 1992). Optimizations were made assuming Fitch parsimony, equal weighting of all characters, transitions among all states equally probable and characters as unordered. Character states were determined from literature and personal observations. Full sample of the eight Cistaceae genera is not presented, except for *Cistus* and *Halimium*. Instead, we chose species representatives of either key character that were coded as particular or polymorphic states (i. e. we did not analyse color states in *Helianthemum* because sampling was limited and the character has three states, but used a polymorphic coding).

3. Results

3.1. Phylogenetic relationships

Characteristics of the two sequence data sets are summarized in Table 4. Comparison of the Cistaceae sequence pairs across the *trnL-trnF* spacer give divergence values ranging from 0.0% (between five conspecific accessions and between *Hel. almeriense–Hel. scopulicolum, Hel. aegyptiacum–Hel. ledifolium, Hel. marifolium–Hel. oelandicum, Hal. lasiocalycinum–Hal. lasianthum* subsp. *alyssoides, Hal. halimifolium–Hal. lasianthum* subsp. *alyssoides, Hal. halimifolium–Hal. lasiocalycinum, C. horrens–C. ochreatus, C. symphytifolius–C. chinamadensis, C. creticus–C. albidus, C. clusii–C. munbyi*) to 25.8% (*Tuberaria guttata–Fumana ericoides*) using the GTR model of evolution. *rbcL* sequence divergence ranges from 0.0% (between six conspecific accessions, between Canarian *Cistus* accessions, *Hel. squamatum–Hel. scopulicolum, Hel. argenteum–Hel. pringlei, Hal. lasiocalycinum–Hal.*

atlanticum, Hal. halimifolium–Hal. lasianthum, Hal. halimifolium–Hal. lasiocalycinum, C. creticus–C. albidus, C. heterophyllus–C. albidus, C. heterophyllus–C. creticus, C. monspeliensis–C. populifolius, C. pouzolzii–C. populifolius, C. pouzolzii–C. monspeliensis, C. albanicus–C. populifolius) to 4.74% (*Tuberaria guttata–Helianthemum oelandicum*).

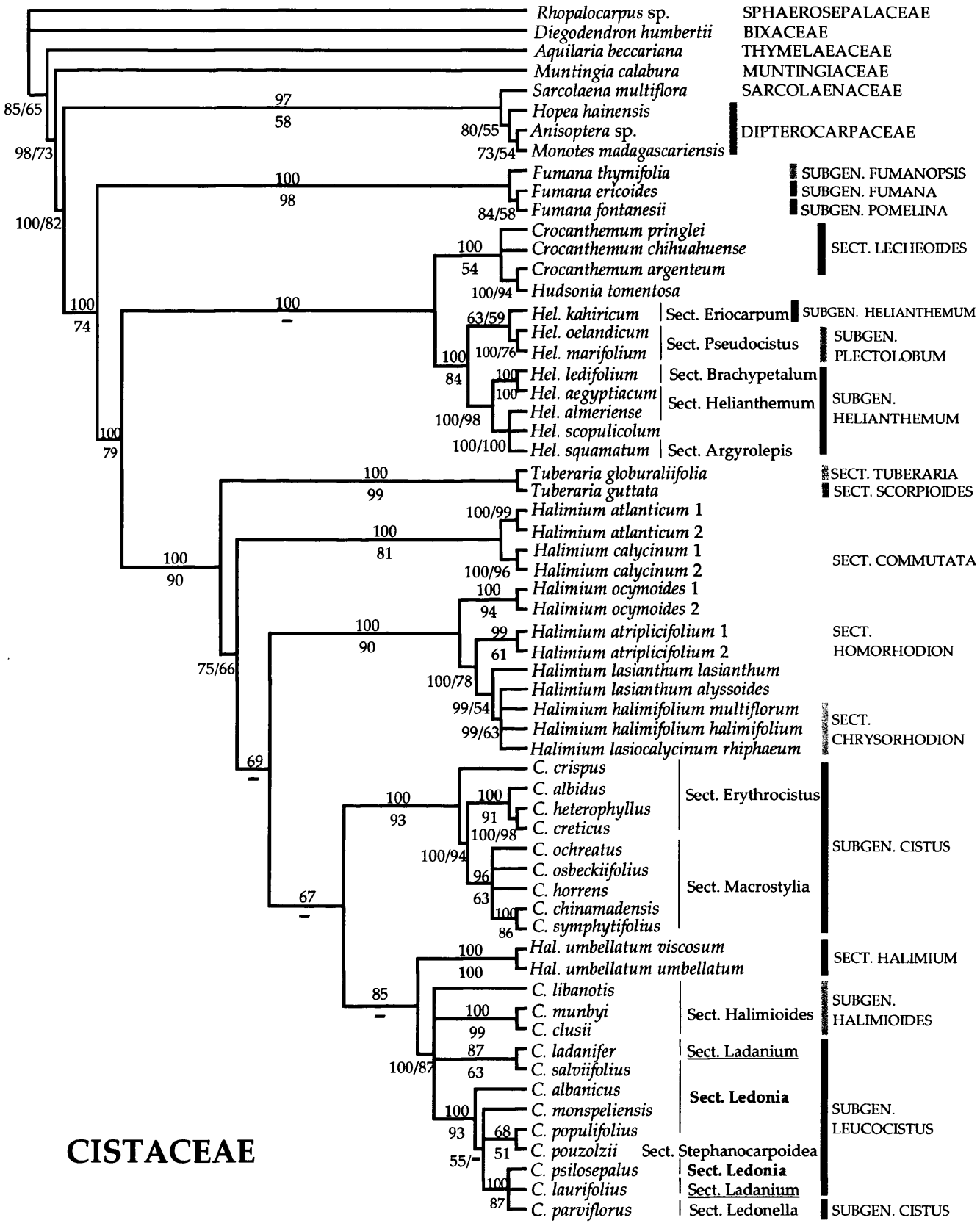
Table 4. Phylogenetic characteristics obtained from the analysis of *trnL-trnF* and *rbcL* sequences of the Cistaceae

	trnL-trnF	*rbcL*
Length (bp)		
Total aligned length	519	1405
Length range - outgroup	399-409	1405
Length range - ingroup	324-460	1404-1405
Number of characters		
Total included	519	1365
Variable/parsimony-informative	181/118	157/108
Maximum sequence divergence (GTR)	25.8%	4.47%
Mean G+C content	32%	54%

The combined data matrix of the Cistaceae plus outgroup sequences consists of 1884 characters, of which 550/341 are variable/parsimony-informative. The heuristic search resulted in 29,678 equally parsimony trees, each with a length of 932 steps, a Consistency Index (CI) of 0.73 and a Retention Index (RI) of 0.86. The BI analysis of the combined data matrix (*rbcL, trnL-F*) (Table 3) displays better resolution (Fig. 1) than the strict consensus tree (result not shown). Both analyses depict the Cistaceae as a monophyletic family (100 PP, 74% BS), sister to the tropical families Dipterocarpaceae and Sarcolaenaceae (Fig. 1). Within the Cistaceae clade, *Fumana* consistently has a

→

Fig. 1. Phylogeny of the Cistaceae and other representatives of Malvales as inferred with Bayesian Inference analysis using sequences from the plastid *rbcL* exon and *trnL-F* spacer. Numbers above branches are posterior probabilities. Numbers bellow branches show bootstrap values in a MP analysis mostly congruent with this tree. Taxonomy follows Demoly and Montserrat (1993) in *Cistus*, Nogueira *et al.* (1993) in *Halimium*, Willkomm (1856) in *Tuberaria*, López (1993) and Grosser (1903) in *Helianthemum*, Arrington & Kubitzski (2003) in *Crocanthemum*, Güemes & Molero (1993) in *Fumana*.


Rhopalocarpus sp.
Diegodendron humbertii
Aquilaria beccariana
Muntingia calabura
Sarcolaena multiflora
Hopea hainensis
Anisoptera sp.
Monotes madagascariensis
Fumana thymifolia
Fumana ericoides
Fumana fontanesii
Crocanthemum pringlei
Crocanthemum chihuahuense
Crocanthemum argenteum
Hudsonia tomentosa
Hel. kahiricum
Hel. oelandicum
Hel. marifolium
Hel. ledifolium
Hel. aegyptiacum
Hel. almeriense
Hel. scopulicolum
Hel. squamatum
Tuberaria globuraliifolia
Tuberaria guttata
Halimium atlanticum 1
Halimium atlanticum 2
Halimium calycinum 1
Halimium calycinum 2
Halimium ocymoides 1
Halimium ocymoides 2
Halimium atriplicifolium 1
Halimium atriplicifolium 2
Halimium lasianthum lasianthum
Halimium lasianthum alyssoides
Halimium halimifolium multiflorum
Halimium halimifolium halimifolium
Halimium lasiocalycinum rhiphaeum
C. crispus
C. albidus
C. heterophyllus
C. creticus
C. ochreatus
C. osbeckiifolius
C. horrens
C. chinamadensis
C. symphytifolius
Hal. umbellatum viscosum
Hal. umbellatum umbellatum
C. libanotis
C. munbyi
C. clusii
C. ladanifer
C. salviifolius
C. albanicus
C. monspeliensis
C. populifolius
C. pouzolzii
C. psilosepalus
C. laurifolius
C. parviflorus
SPHAEROSEPALACEAE
BIXACEAE
THYMELAEACEAE
MUNTINGIACEAE
SARCOLAENACEAE
DIPTEROCARPACEAE
SUBGEN. FUMANOPSIS
SUBGEN. FUMANA
SUBGEN. POMELINA
SECT. LECHEOIDES
Sect. Eriocarpum
SUBGEN. HELIANTHEMUM
Sect. Pseudocistus
SUBGEN. PLECTOLOBUM
Sect. Brachypetalum
Sect. Helianthemum
SUBGEN. HELIANTHEMUM
Sect. Argyrolepis
SECT. TUBERARIA
SECT. SCORPIOIDES
SECT. COMMUTATA
SECT. HOMORHODION
SECT. CHRYSORHODION
Sect. Erythrocistus
SUBGEN. CISTUS
Sect. Macrostylia
SECT. HALIMIUM
Sect. Halimioides
SUBGEN. HALIMIOIDES
Sect. Ladanium
Sect. Ledonia
SUBGEN. LEUCOCISTUS
Sect. Stephanocarpoidea
Sect. Ledonia
Sect. Ladanium
Sect. Ledonella
SUBGEN. CISTUS
CISTACEAE

basalmost position (100 PP, 79% BS). Although this result should be taken with caution due to the lack of *Lechea* representatives in our phylogenetic analysis (Fig. 1), unpublished results by Arrington & Kubitzki (2003) and a recent addition of one sample of *Lechea tripetala* obtained in our laboratories (November, 25, 2007) confirm the sister group condition of *Fumana* to the rest of the Cistaceae, followed by an unresolved trichotomy of two clades as in Figure 1 plus one more of the sample of *Lechea*. Only BI clearly supports (100 PP) the sister relationship of the New World genera *Crocanthemum* and *Hudsonia* with the Old World genus *Helianthemum*, whereas both analyses strongly agree with the sister-group relationship of the clade of these three genera with the rest of the Cistaceae (*Tuberaria, Halimium, Cistus*) (100 PP, 90% BS). A very close relationship between American genera can be predicted by the strong grouping of *Hudsonia tomentosa* and *Crocanthemum argenteum* (100 PP, 94% BS). The phylogenetic analyses reveal the infrageneric representative accessions of *Helianthemum* as a well-supported monophyletic lineage (100 PP, 84% BS) (Fig. 1). *Tuberaria* appears to be the sister group to *Cistus* and *Halimium* but only with low support in the BI (75 PP). Both analyses indicate monophyly of conspecific accessions of *Halimium atlanticum* (100 PP; 99% BS), *H. calycinum* (100 PP; 96% BS), *H. umbellatum* (100 PP; 100% BS) and *H. ocymoides* (100 PP; 94% BS). *Halimium* species are divided in three supported subclades (Fig. 1) although relationships among subclades are resolved but weakly supported in the BI and not resolved in the MP. *Cistus* accessions form a monophyletic group as long as *H. umbellatum* is included. Only two well-supported clades of the purple-flowered (excluding *C. parviflorus*) (100 PP, 93% BS) and the white-flowered (100 PP, 87%BS) *Cistus* species are retrieved. The Bayesian inference analysis depicts the *Halimium umbellatum* group (subspp. *umbellatum, viscosum)* sister to the white-flowered lineage of *Cistus* (100 PP), while the MP analysis does not recognized this relationship.

3.2. Patterns of character evolution

The most significant characters (14) in the classification of the Cistaceae are mapped on the MP tree in agreement with the Bayesian analysis to investigate patterns of evolution. Admittedly, the only missing genus (*Lechea*) in the analysis may provide an

alternative hypothesis to character reconstruction at basal levels of the Cistaceae. We however consider that some of the results are equally valid irrespective of using the placement of *Lechea* data in the analysis (see above). The most relevant results from the historical reconstructions are following described:

1. Life form (Fig. 2A). The character is revealed as very homoplastic within the family. The shrub form appeared up to five times in the *Cistus-Halimium* complex while the herbaceous form (perennial and annual herbs) arose independently in three genera (*Crocanthemum, Helianthemum, Tuberaria*). The subshrub form appears to be plesiomorphic and maintained in the eight genera of the Cistaceae.

2. Petal color (Fig. 2B). The character state reconstruction shows yellow flowers as plesiomorphic. Purple flowers evolved twice in *Cistus* while only one event was necessary to originate white flowers in the *Cistus-Halimium* assemblage.

3. Petal macule (Fig. 2C). Recurrent acquisition of the character in *Helianthemum, Halimium* and *Tuberaria* can be interpreted in the character reconstruction. In *Cistus,* only *C. ladanifer* (white-flowered lineage) displays some populations with a marked macule (see appendix 1).

4. Sepal number (Fig. 2D). A calyx with 5 sepals has been maintained mostly in the stem groups of the Cistaceae. Evolution of this character has however been dynamic in the group of *Tuberaria-Halimium-Cistus.* For instance, character optimization depicted three sepals recurrently at least twice in *Cistus.*

5. Carpel number (Fig. 2E). Increment of carpel number was clearly observed in the Cistaceae. The reconstruction is however equivocal for the 5-carpellated ovary of *Cistus* and the 3-carpellated ovary of *Halimium umbellatum.* Ovaries with 5-12 carpels are only found in *C. ladanifer,* which originated only once bringing about the higher number of fruit divisions in the Cistaceae.

6. Embryo shape (Fig. 2F). The basal-most lineage of *Fumana* shares the ancestral curved embryo with the American genera (Arrington & Kubitzki 2003). The embryo

shape is however equivocal for the *Halimium-Cistus* ancestor, although *Cistus* displays only circinate embryos.

7. Ovule position (Fig. 2G). The reconstruction of character states depicts *Fumana* as the only genus in the family retaining the plesiomorphic state (anatropous ovules).

8. Staminodes (Fig. 2H). The presence of staminodes in the periphery of the androecium is revealed as a very polymorphic character within Malvales. In the Cistaceae, this character state is only found in *Fumana*.

9. Pollen-type (Fig. 2I). Traced of the five pollen types depicts consistency with previous proposals (Ukraintseva 1993) both in the *Tuberaria-Halimium-Cistus* group (*Cistus*-type) and in *Helianthemum* (*Helianthemum*-type), which retains the plesiomorphic state in the Cistaceae. Two pollen types are found in *Fumana*, including the plesiomorphic state.

10. Leaf arrangement (Fig. 2J). The historical reconstruction is equivocal at the base of the Cistaceae phylogeny and does not clarify the evolutionary change at early stages. Acquisition of opposite leaves once in the *Tuberaria-Halimium-Cistus* and *Helianthemum* groups is unequivocal.

11. Haploid chromosome number (Fig. 2K). The gametophytic number of n=10 is common within a basal grade of the Cistaceae (*Crocanthemum, Helianthemum, Hudsonia*). Interestingly, the stem group of *Fumana* has consistently a higher number (n=16), while the crown-group including all the species of the *Cistus-Halimium* complex contains a low number (n=9). This, together with intermediate numbers between the two lineages, suggests a general trend to chromosome number reduction in the Cistaceae (but see n=7 in *Helianthemum* and n=5 in *Tuberaria* as exceptions).

12. Distribution (Fig. 2L). Considering the dataset herein provided, an Old World ancestry of the Cistaceae is inferred by genera distribution optimization. Given the mid-term position of the *Lechea* sample in the Cistaceae phylogeny (unpublished data), our results agree with an early center of diversification in the Mediterranean-European

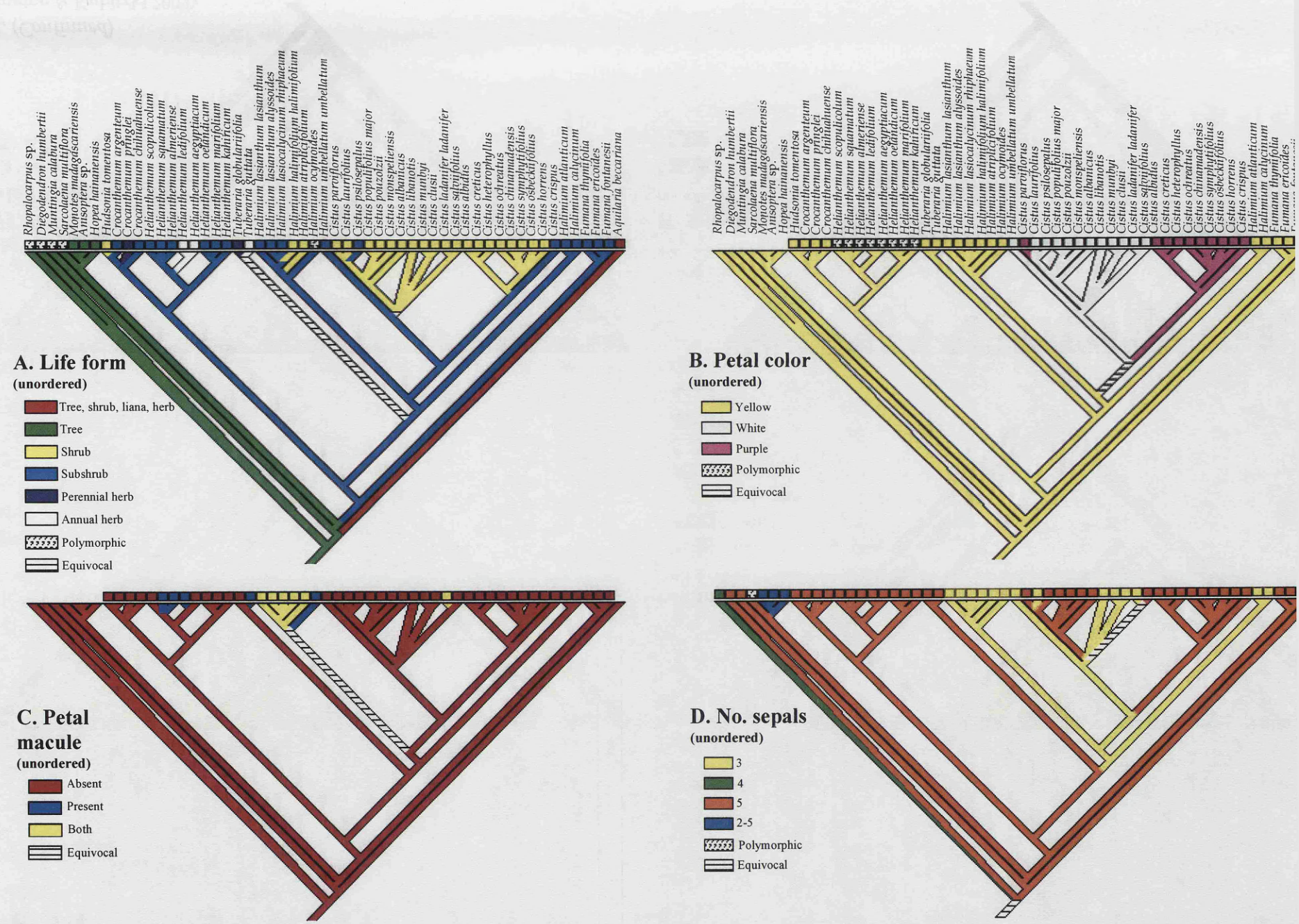
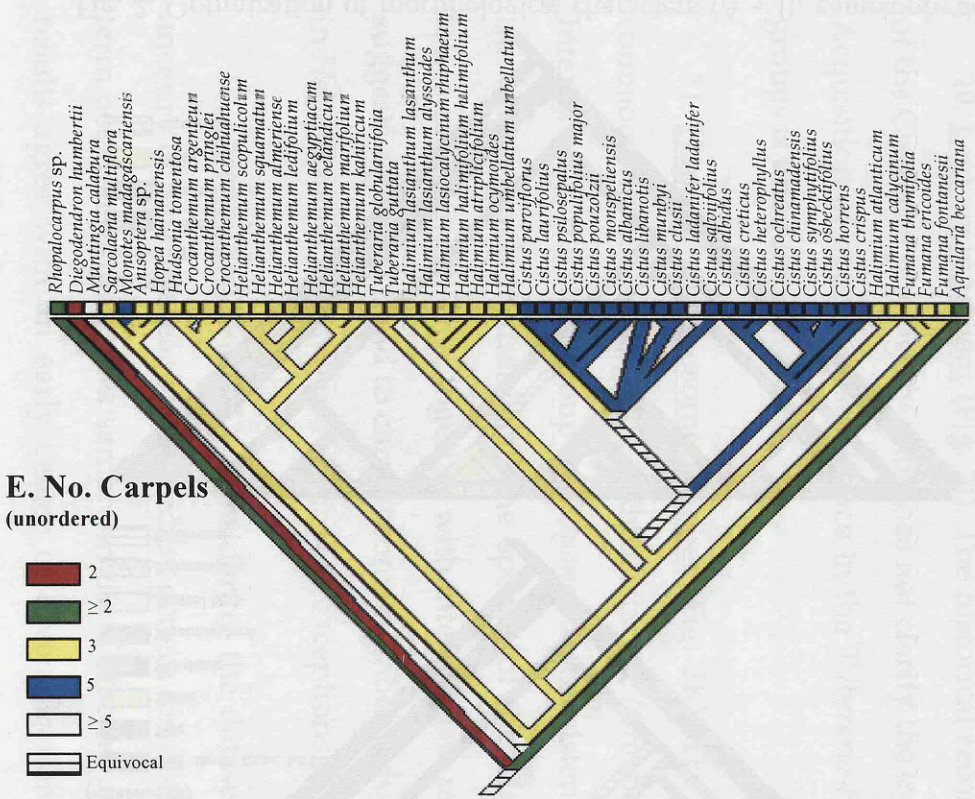
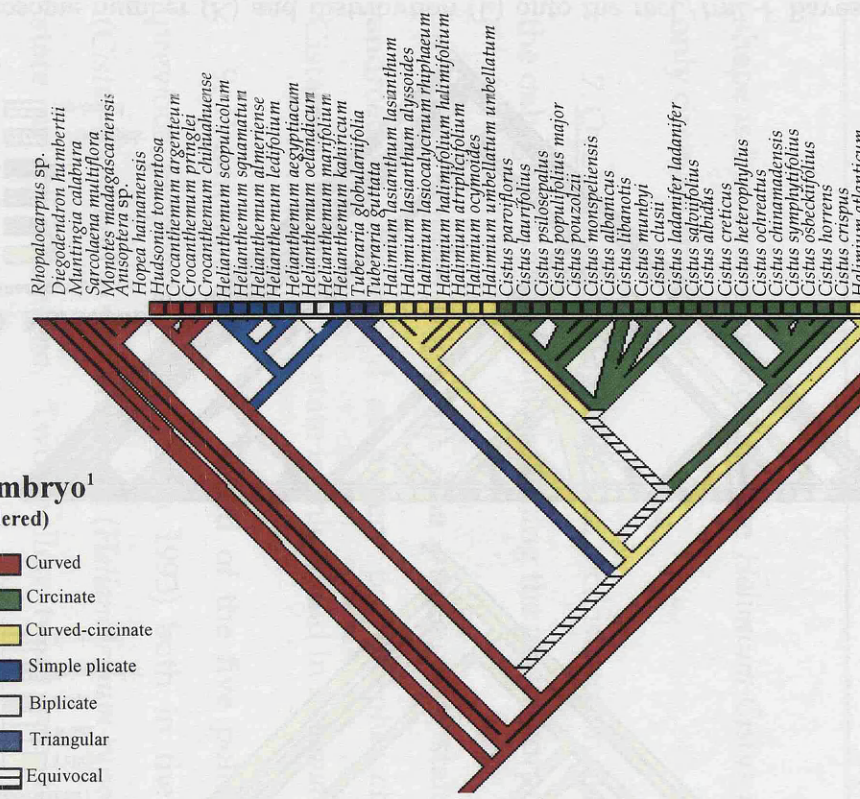
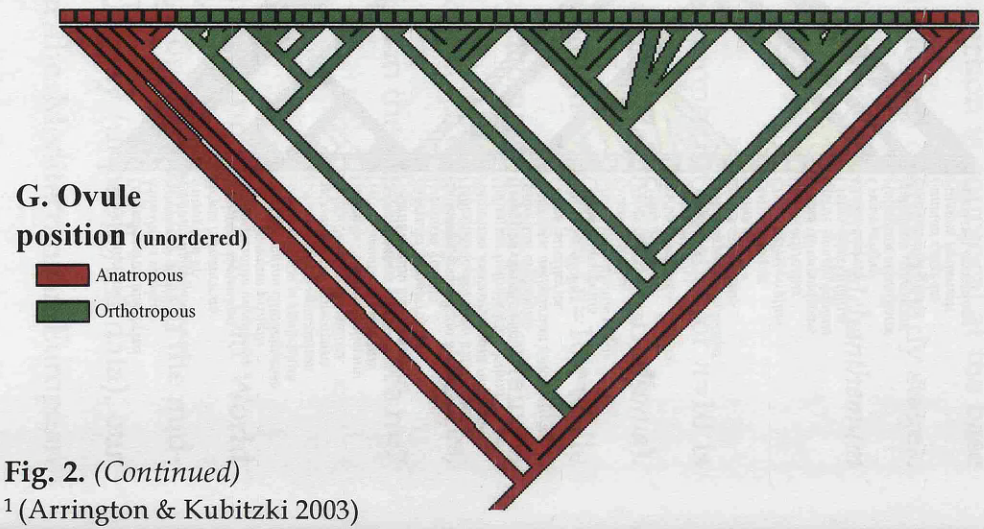


Fig. 2. Optimization of morphological characters (A – J), gametophytic chromosome number (K) and distribution (L) onto the *rbcL/trnL-F* Bayes inference tree.







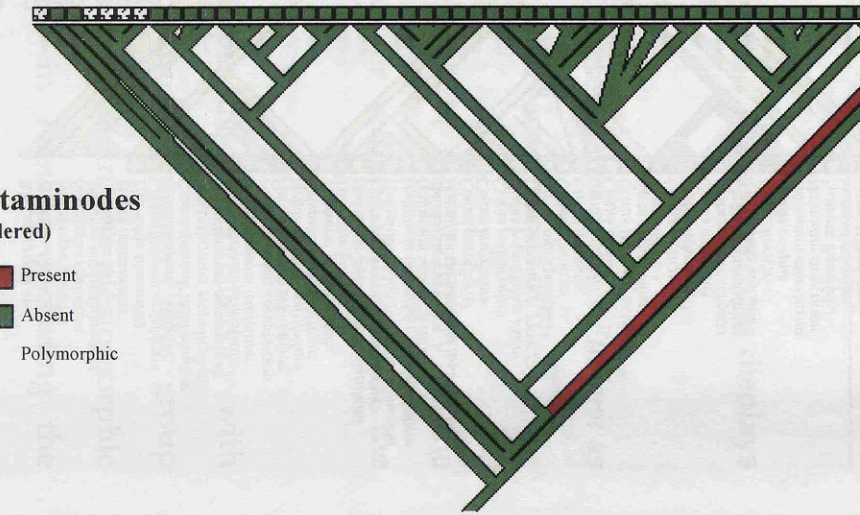
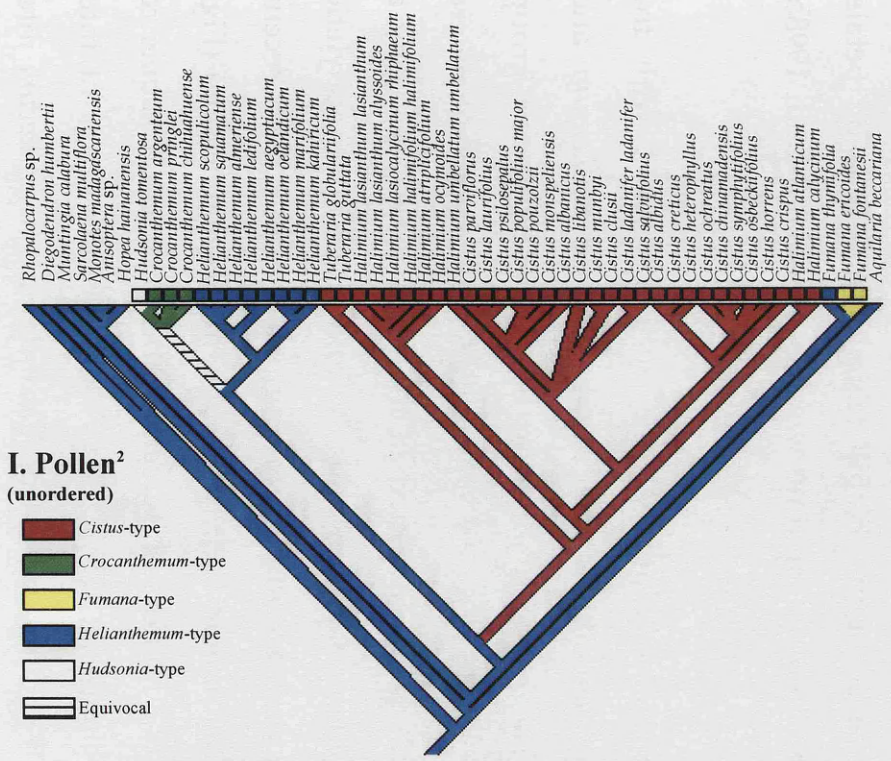
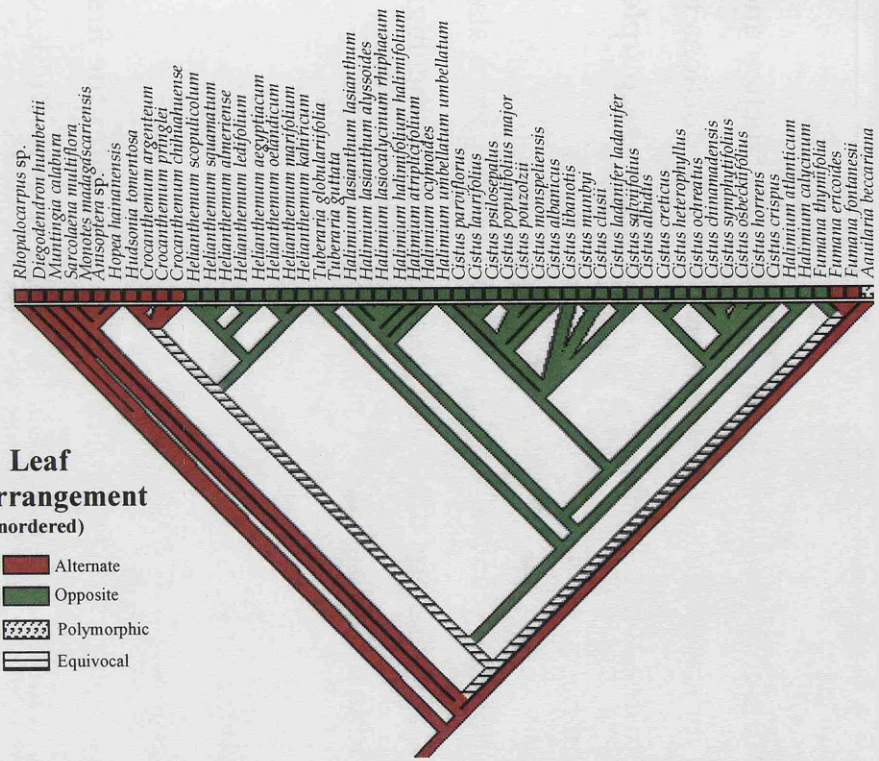
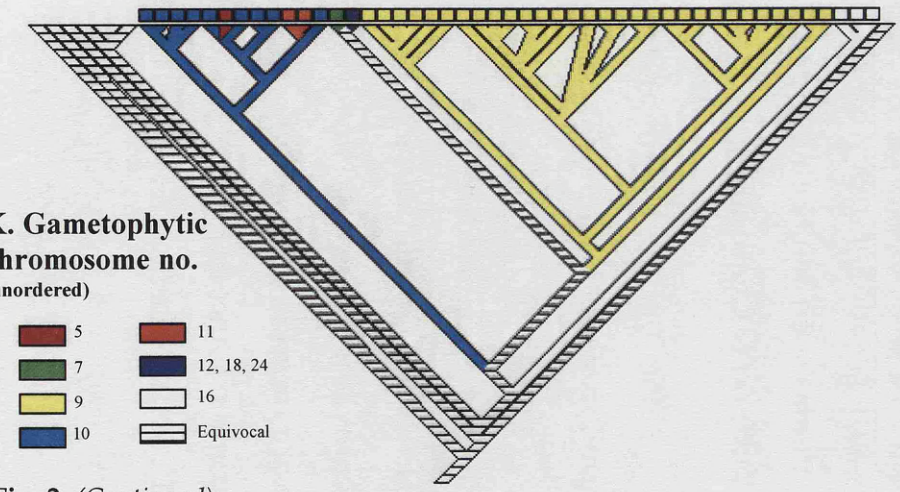


Fig. 2. *(Continued)*

[1] (Arrington & Kubitzki 2003)







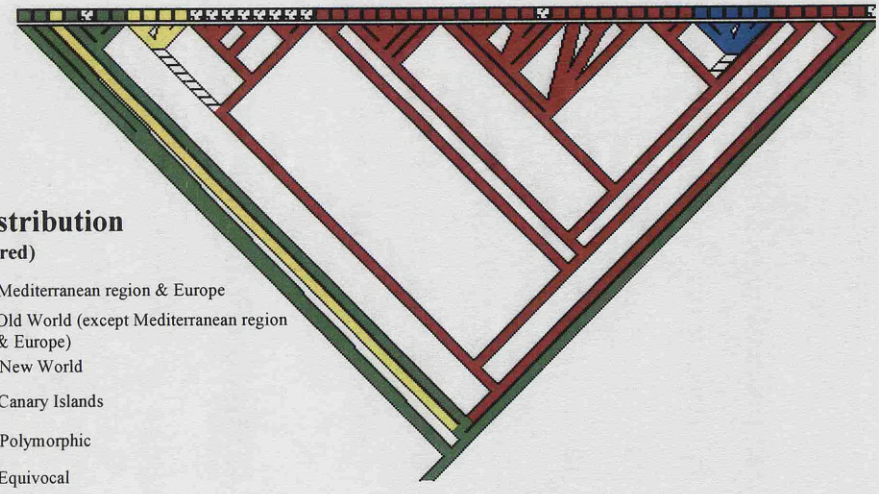


Fig. 2. *(Continued)*

[2] Ukraintseva (1993)

region because of the basal-most position of *Fumana*. Our character reconstruction and the occurrence of endemic species indicate at least three independent introductions to the Canarian archipelago: one from the white-flowered *Cistus*, one from the purple-flowered *Cistus* and at least one from *Helianthemum*.

Leaf attachment (stipulate, exstipulate) and leaf base (petiolate, sessile) were also reconstructed (results not shown) but interpretation was difficult due to the high variability of the characters even within a single species.

4. Discussion

4.1. Phylogenetic relationships in the Cistaceae

Our study, based on the plastid *rbcL* gene and the *trnL-trnF* spacer, provides the first available phylogenetic framework within the family (but see Arrington & Kubitzki 2003). MP and BI analyses recognize the Cistaceae as a well defined, monophyletic group also defined by some morphological characters (parietal placentation, lack of mucilage and/or resin canals, presence of multipapillate epidermal cells on petals, stigmas with multicellular papillae, dimorphic and no wing-like sepals) (Nandi 1998b; Kubitzki & Chase 2003).

None of the classifications proposed (Table 2) are fully congruent with the phylogenetic hypothesis herein presented because the genera *Crocanthemum* and *Halimium* are not monophyletic. The historical division of the *Helianthemum* group (highly supported as monophyletic; Fig. 1) into two infrageneric taxa (subgenera *Helianthemum, Plectolobum*) (Table 2) is also depicted in the phylogeny (Fig. 1) by a biphyletic topology. However, *Helianthemum* subgenus *Helianthemum* is paraphyletic because the *Helianthemum* subgenus *Plectolobum* group originated from a most recent common ancestor shared with *Helianthemum kahiricum* (subgenus *Helianthemum*) (Fig. 1). At the section level, we have not found full agreement between DNA sequence variation and morphology of *Helianthemum* (López 1993). Our results better fit into Dunal's (1824) and Grosser's (1903) circumscription of *Helianthemum aegyptiacum* into the section *Brachypetalum*. Neither historical nor recent classifications (Table 2, Fig. 1)

recognise a division of three sections of *Halimium* in agreement with the three monophyletic groups retrieved (Fig. 1). Only Jiménez's (1981) delimitation into three sections agrees with our results (although African species were not included in her study): sect. *Halimium* (*H. umbellatum*); sect. *Chrysorhodion* Spach (*H. atriplicifolium, H. halimifolium, H. lasianthum, H. ocymoides*); sect. *Commutatae* (*H. calycinum*). Additionally, our phylogenetic hypothesis indicates that paraphyly also affects the two subspecies accessions of *Halimium lasianthum* (Fig. 1). The naturalness of the *Cistus-Halimium* assemblage suggested in previous studies (Guzmán & Vargas 2005) is herein confirmed, supporting somehow the statement that "parallel evolution with occasional junctions (and maybe character exchanges)" for some characters pointed out by Dansereau (1939).

Extensive work (sampling and sequencing) is certainly required to fully elucidate the phylogenetic relationships of the Cistaceae. Lack of resolution in some clades makes necessary to extend DNA sequencing, particularly from the nucleus, to generate a more consistent evolutionary hypothesis. Additional accessions of limited sampled genera in general and of the key genus *Lechea* are also needed to obtain a complete phylogenetic hypothesis of the extant Cistaceae.

4.2. Nandi's hypothesis of Fumana *as a basal lineage*

Two studies analysed in detail the evolution of ontogenetic patterns in the Cistaceae and related Malvales (Nandi 1998a, 1998b). The comparison of floral development in Malvales families revealed similarities only found in one genus (*Fumana*) of the Cistaceae. For instance, the ovule position is anatropous in *Fumana*, as in related Malvales, while orthotropous in the rest of the Cistaceae. Moreover, *Fumana procumbens* shows a stigma shape similar to that found in floral buds of some Sarcolaenaceae. Only the seed is the diaspora in the Cistaceae, except for *Fumana procumbens* and *F. baetica* which are dispersed by fruits (with the contribution of the calyx) (Hegi 1925). Similarly, seed dispersal by fruits is also shown in Dipterocarpaceae (Ashton 2003). Based on these flower and fruit morphological features Nandi suggested "an isolated position of *Fumana* at the base of the Cistaceae".

A historical inference of character state transformations allows us testing Nandi's hypothesis of character evolution. Although *Lechea* and some key species are missing in our *rbcL/trnL-F* tree (Fig. 1), all the analyses are consistent with a basal position of *Fumana* (Fig. 1). This result is also congruent with the phylogenetic hypothesis based on plastid and nuclear DNA sequences described in Arrington & Kubitzski (2003) and our most-recent analysis including *Lechea tripetala* (results not shown). One of the most reliable phylogenetic hypothesis help reconstruct character-state evolution (Fig. 2). The inference of the anatropous ovule position does not only support an early branching of the *Fumana* lineage, but also character retention (Fig. 2G). Nandi's hypothesis was also based in Ukraintseva's (1993) Cistaceae pollen classification. Ukraintseva described six types of pollen mostly congruent with genera subdivision of the family (only *Tuberaria, Halimium* and *Cistus* share the same pollen-type). The sister-group relationship between *F. thymifolia* and two other *Fumana* species (although weakly support; 84 PP, 58% BS) also suggested plesiomorphy for the *Helianthemum* pollen-type (Fig. 2I). Nevertheless, the polymorphic state of the character in *Fumana* makes necessary the analysis of all the species. The sharing of a key character state with some relatives is not however sufficient to assume a single origin from a common ancestor. Nandi (1998a) indeed pointed out that the presence of staminodes in the periphery of the androecium in *Fumana* (Cistaceae), *Xyloolaena* (Sarcolaenaceae) and *Dipterocarpus* (Dipterocarpaceae) may be consider as close parallelisms. Our character state optimization (Fig. 2H) confirms that the presence of staminodes is a synapomorphy of *Fumana* in the Cistaceae, but recurrent in the Malvales. Similarly, staminodes evolved independently at least 14 times within the Rosidae (Walker-Larsen & Harder 2000).

In conclusion, Nandi's hypothesis of an isolated position of *Fumana* at the base of the Cistaceae is a plausible prediction, providing that addition of *Lechea* and other genera species does not alter significantly the spine of the tree. Stigma and diaspore traits should be also investigated in a wider number of genera and species to infer whether ancestral states of these two characters are also retained in *Fumana*.

4.3. *Differentiation of the Mediterranean Cistaceae*

The incomplete sample of the American genera (*Lechea* and *Crocanthemum* section *Spartioides*) and topological irresolution at some clades in the MP analysis for the *Crocanthemum-Hudsonia* and *Helianthemum* relationship prevented from addressing some key questions in the evolution of the Cistaceae: the number of migration events between the New and the Old World, and the origin of the trimery in petals, stigmas and stamens found in *Lechea*[*].

The location of the most ancestral forms can be used to infer the geographical origin of a taxon (Platnick 1981). Both MP and BI analyses suggest an early Mediterranean-European divergence of the Cistaceae. Within the family, the basal-most lineage found so far (*Fumana*, Fig. 1) is exclusive to the Mediterranean (although *F. procumbens* is distributed in central Europe and Euroasiatic regions, Güemes & Molero 1993). A Middle Oligocene macrofossil from Germany, described as an ancestor of the extant Cistaceae (*Cistinocarpum roemeri*) (Palibin 1909), and *Tuberaria* pollen found in Pliocene formations of Germany (Menke 1976) as the oldest fossil records suggest an ancient presence of the family in Europe, but ranging out of the current centre of diversification (the western Mediterranean region) (Guzmán & Vargas 2005). An ancient Cistaceae occurrence in Europe is further supported by the genera distribution optimization (Fig. 2L). As many other Mediterranean species, the Cistaceae appears to have differentiated in tropical areas occurring in the Tertiary (Herrera 1992). Fossil evidence, the Eocene-Oligocene split between Sarcolaenaceae-Dipterocarpaceae and Cistaceae lineages (Wikström *et al.* 2001) and a dominant tropical vegetation in Europe (Palamarev 1989) suggest a Mid Tertiary origin of Cistaceae in the Old World. Additionally, the Mediterranean Basin harbors not only the highest number of species (97) and genera (5), but also the fundamental lineages of the Cistaceae, which have been argued as strong argument for ancient biodiversity (Forest *et al.* 2007). An origin inference may be

[*] The new analysis not included in this dissertation is congruent with an early Mediterranean center of diversity for the Cistaceae and to consider trimery in petals, stamens and stigmas autoapomorphies of *Lechea*. However, the lack of resolution between *Lechea* and the rest of the Cistaceae genera prevented from estimating the number of migration events to the New World.

however cautiously considered given missing taxa in our analysis and the solid arguments discussed on the centre of origin and centre of diversification (Bremer 1992). In any case, the biogeographic reconstruction herein presented (Fig. 2L) is consistent with an early (primary or secondary) differentiation process of the Cistaceae in the Mediterranean, and then divergence of the New World genera (*Hudsonia, Crocanthemum, Lechea*).

The close relationship of three Old World genera (*Tuberaria, Halimium, Cistus*), as previously recognized (Ukraintseva 1993; Nandi 1998a), is one of the most robust findings of the present study (Fig. 1, Fig. 2I). The present data, coupled with previous analysis of plastid (*trnL-F, trnK-matK*) and nuclear (ITS) sequences (Guzmán & Vargas 2005), show a congruent topology in which *Cistus* is imbedded in *Halimium*. A cohesive evolutionary history of the *Cistus-Halimium* complex may have primarily occurred in the Mediterranean region since two of the three genera and 29 of c. 45 species are currently exclusive to the Mediterranean Basin. Multiple shifts in chromosome numbers reflect active cytological differentiation in the Cistaceae (Fig. 2K), but not in the *Cistus-Halimium* complex. *Halimium* and *Cistus* divergence partnered no change neither in the chromosome number (n=9) nor in a predominant self-incompatibility mechanism (Carrió *et al.* 2003). In this group, historical reconstruction of petal color recognizes character retention in one white-flowered *Halimium* (*H. umbellatum*) and the white-flowered *Cistus* vs. acquisition and maintenance of mauve petals in the purple-flowered lineage (Fig. 2B), despite intensive selective pressure on flower color in entomophylous angiosperms (Irwin & Strauss 2005).

In summary, a significant number of characteristics (leaf arrangement, embryo shape, ovule position, carpel number, staminode presence, pollen types) appear to have been conserved in the course of Cistaceae evolution, whereas life form, sepal number, petal macules and chromosome number have been more dynamic.

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References

Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap? a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**, 255-266.

Alverson WS, Karol KG, Baum DA, *et al.* (1998) Circumscription of the Malvales and relationships to other Rosidae: evidence from *rbcL* sequence data. *American Journal of Botany* **85**, 876-887.

Arrington JM, Kubitzki K (2003) Cistaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 62-70. Springer, Berlin.

Ashton PS (2003) Dipterocarpaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 182-195. Springer, Berlin.

Baas P, Werker E (1981) A new record of vestured pits in Cistaceae. *IAWA Bulletin new series* **II**, 41-42.

Bremer K (1992) Ancestral areas: A cladistic reinterpretation of the center of origin concept. *Systematic Biology* **41**, 436-445.

Calderón G (1992) Cistaceae. *Flora del Bajío y de regiones adyacentes* **2**, 1-27.

Carrió E, Herreros R, Güemes J (2003) Breeding system evolution in Mediterranean Cistaceae. In: *IXth IOPB Meeting. Plant evolution in Mediterranean climate zones*, Valencia. Spain.

Corner EJH (1976) *The seeds of dicotyledons.* Cambridge University Press, Cambridge.

Cronquist A (1988) *The evolution and classification of flowering plants.* New York Botanical Garden, Bronx, NY.

Dahlgren G (1989) The last Dahlgrenogram: system of classification of the Dicotyledons. In: *Plant taxonomy, phytogeography and related subjects* (eds. Tan K, *et al.*), pp. 249-260. Edinburgh University, Edinburgh.

Dansereau P (1939) Monographie du genre *Cistus*. *Boissiera* **4**, 1-90.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Ducousso M, Béna G, Bourgeois C, *et al.* (2004) The last common ancestor of Sarcolaenaceae and Asian dipterocarp trees was ectomycorrhizal before the India-Madagascar separation, about 88 million years ago. *Molecular Ecology* **13**, 231-236.

Dunal (1824) Cistineae in De Candolle. A. P. *Prodromus Systematis Naturalis Regni Vegetabilis* **I**, 263-286.

Fay MF, Bayer C, Alverson WS, de Brujin AY, Chase MW (1998) Plastid *rbcL* sequence data indicate a close affinity between *Diegodendron* and *Bixa*. *Taxon* **47**, 43-50.

Fernald ML (1917) *Helianthemum dumosum* on the mainland of New England. *Rhodora* **19**, 58-60.

Forest F, Grenyer R, Rouget M, *et al.* (2007) Preserving the evolutionary potential of floras in biodiversity hotspots. *Nature* **445**, 757-760.

Gamage DT, de Silva MP, Inomata N, Yamazaki T, Szmidt AE (2006) Comprehensive molecular phylogeny of the sub-family Dipterocarpoideae (Dipterocarpaceae) based on chloroplast DNA sequences. *Genes and genetic system* **81**, 1-2.

Grosser W (1903) Cistaceae. In: *Dans Pflanzenreich* (ed. Engler A), p. 161, Berlin.

Güemes J, Molero J (1993) *Fumana*. In: *Flora ibérica* (eds. Castroviejo S, *et al.*), pp. 422-436. Consejo de Investigaciones Científicas, Madrid.

Guzmán B, Vargas P (2005) Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trn*L-*trn*F, and *mat*K sequences. *Molecular Phylogenetics and Evolution* **37**, 644-660.

Hegi G (1925) *Illustrierte Flora von Mitteleuropa*, München: Lehmann.

Herrera C (1992) Historical effects and sorting processes as explanations for contemporary ecological patterns: character syndromes in Mediterranean woody plants. *The American Naturalist* **140**, 421-446.

Horn JW (2004) The morphology and relationships of the Sphaerosepalaceae (Malvales). *Botanical Journal of the Linnean Society* **144**, 1–40.

Irwin RE, Strauss SY (2005) Flower color microevolution in wild radish: Evolutionary response to pollinator-mediated selection. *The American naturalist* **165**, 225-237.

Jiménez MJ (1981) *Taxonomía del género Halimium (Dun.) Willk. (Cistaceae)*, Universidad Complutense de Madrid.

Kubitzki K, Chase MW (2003) Introduction to Malvales. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 12-16. Springer, Berlin.

Linnaeus C (1753) *Species Plantarum*, Estocolmo.

López G (1993) *Helianthemum*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 365-421. Consejo de Investigaciones Científicas, Madrid.

Maddison WP, Maddison DR (1992) *MacClade: Analysis of Phylogeny and Character Evolution, versión 3.01*. Sinauer, Sunderland, MA.

Martín Bolaños M, Guinea E (1949) *Jarales y Jaras (Cistografia Hispanica)*. Ministerio de Agricultura. Direccion General de Montes, Caza y Pesca Fluvial. Instituto Forestal de Investigaciones y Experiencias, Madrid.

Menke B (1976) Pliozäne und ältestquartäre Sporen- und pollenflora von Schleswig-Holstein. *Geologisches Jahrbuch. Reihe A.* **32**, 3-197.

Mort ME, Soltis PS, Soltis DE, Mabry M (2000) Comparison of three methods for estimating internal support on phylogenetic trees. *Systematics Biology* **49**, 160-171.

Nandi OI (1998a) Floral development and systematics of Cistaceae. *Plant Systematics and Evolution* **212**, 107-134.

Nandi OI (1998b) Ovule and seed anatomy of Cistaceae and related Malvanae. *Plant Systematics and Evolution* **209**, 239-264.

Nogueira I, Muñoz Garmendia F, Navarro C (1993) *Halimium*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 337-351. Consejo de Investigaciones Científicas, Madrid.

Nylander JAA (2002) MrModeltest v1.0b. Department of Systematic Zoology. Uppsala University, Uppsala.

Palamarev E (1989) Palebotanical evidences of the Tertiary history and origin of the Mediterranean sclerophyll dendroflora. *Plant Systematic and Evolution* **162**, 93-107.

Palibin IV (1909) Cistaceae. In: *Flora Caucasica Critica* (eds. Kuznetsov N, *et al.*), pp. 117-123.

Platnick NI (1981) The progression rule or progression beyond rules. In: *Vicariance biogeography - a critique* (eds. Nelson G, Rosen DE), pp. 144-150. Columbia University Press, New York, USA.

Ponzo A (1921) Considerazioni sulle Cistaceae. *Nuovo Giorno Botanoco Italia* **28**, 157-173.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Savolainen V, Chase MW, Hoot SB, *et al.* (2000) Phylogenetics of flowering plants based on combined analysis of plastid *atpB* and *rbc*L gene sequences. *Systematic Biology* **49**, 306-362.

Soltis DE, Soltis PS, Chase MW, *et al.* (2000) Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. *Botanical Journal of the Linnean Society* **133**, 381-461.

Soltis DE, Soltis PS, Endress PK, Chase MW (2005) *Phylogeny and evolution of angiosperms.* Sinauer Associates, Inc., Sunderland, Massachussetts.

Spach E (1836) Conspectus monographiae Cistacearum. *Annales des Sciences Naturelles, Botanique* **6**, 357-375.

Swofford DL (1999) *"PAUP: phylogenetic analysis using parsimony," Version 4.0.* Illinois Natural History Survey, Champaign, Illinois.

Taberlet P, Gielly L, G. P, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105-1109.

Takhtajan A (1987) *Systema magnoliophytorum.* Editoria Nauka, Leningrad (St. Peterburg).

Takhtajan A (1992) Dicotyledones. Dilleniidae. In: *Anatomia seminum comparativa.* Nauka, St. Petersburg.

Takhtajan A (1997) *Diversity and Classification of Flowering Plants,* 2 edn. Columbia University Press, New York.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

Thorne RF (1992) An updated phylogenetic classification of the flowering plants. *Aliso* **13**, 365-389.

Ukraintseva VV (1993) Pollen morphology of the family Cistaceae in relation to its taxonomy. *Grana* **Supplement 2**, 33-36.

Walker-Larsen J, Harder LD (2000) The evolution of staminodes in angiosperms: patterns of stamen reduction, loss, and functional re-invention. *American Journal of Botany* **87**, 1367-1384.

Wikström N, Savolainen V, Chase MW (2001) Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society of London* **268**, 1-10.

Willkomm M (1856) *Cistinearum orbis veteris descriptio monographica. Icones et Descriptiones Plantarum.*

3

Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trnL-trnF*, and *trnK-matK* sequences

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Abstract

This paper presents the first phylogenetic hypotheses for the 20 species of *Cistus* based on plastid (*trnL-trnF, trnK-matK*) and nuclear (ITS) DNA sequence data. Phylogenetic relationships reveal that: (1) *Halimium* and *Cistus* form a cohesive, natural group; (2) two major lineages of purple-flowered and white-flowered species are defined, except for the pinkish-flowered *C. parviflorus;* (3) monophyly of conspecific populations is congruent with the circumscription of species. Topological congruence between nuclear and plastid phylogenies does not support a predominant reticulate system of evolution in *Cistus*. Reconstruction of character evolution suggests an increment of number of fruit valves in the Cistaceae from 3 to 12 in a unidirectional manner. In contrast, reproductive characters, such as sepal number, petal color, and style length, evolved multiple times in the course of evolution. A single colonization of *Cistus* into the Canary Islands appears to be responsible for a lineage of four species sharing a most recent common ancestor with five sepals, purple flowers, styles as long as stamens, and five fruit valves. Species diversity in *Cistus* (14) and *Halimium* (8), coupled with sister-group relationships and molecular divergence, lead us to suggest the western Mediterranean as a major center of present-day differentiation, but paleobotanical data indicates an earlier formation of the *Cistus-Halimium* assemblage in different areas.

Key words: Canary Islands, character evolution, Cistaceae, *Cistus*, ITS, *trnK-matK*, Mediterranean, systematics, *trnL-trnF*

1. Introduction

Cistus (Cistaceae) is one of the most characteristic genera of the Mediterranean flora. Shrubby species primarily occur as woodland understory and others (*C. ladanifer, C. laurifolius, C. monspeliensis*) are dominant in evergreen scrub. The adaptation of the genus to Mediterranean environments is evident from ecological characteristics such as fire-dependent seed germination (Roy and Sonié, 1992; Trabaud and Renard, 1999), insect-dependent pollination (Talavera et al., 1993), flower-dependent reproduction (Herrera, 1987), and spring-dependent phenology (Herrera, 1986). A long history of human activities has favored distribution and abundance of *Cistus* species in the Mediterranean (Thompson, 2005). Impenetrable masses of *Cistus* plants are formed as early successional stages following woodland disturbances such as fire and soil overturning. Co-occurring species of *Cistus* are frequent, particularly in mountain ranges composed by both acidic and basic soils. Environmental specificity referring to substrate confers additional value to acidophilous and basiphilous species as predictable indicators of woodland disturbances. In marked contrast to the detailed knowledge of ecological characteristics of *Cistus*, understanding of the evolution of morphological characters and phylogenetic relationships within the genus is extremely limited.

Cistaceae comprises about 180 species, typically displaying loculicidal capsules of three valves, except in *Cistus* that is characterized by capsules with five or more valves. Circumscription of species in the eight genera of the Cistaceae is still problematic, particularly in genera such as *Helianthemum* and *Halimium*. This has resulted in the publication of multiple combinations for the same taxon under different generic names (Arrington and Kubitzki, 2003). The taxonomy of *Cistus* has traditionally been based on vegetative (nerve number, shape, and hairiness of leaves) and reproductive characters (sepal number, petal color, style length, number of fruit valves). Worldwide monographs of *Cistus* have recognized between 16 species (Grosser, 1903) and 28 species (Dunal, 1824) (Table 1). Following Grosser's (1903) treatment with additional

Table 1. Comparison of historical taxonomic treatments of *Cistus* using taxa names as published in original publications

DUNAL 1824	SPACH 1836	WILLKOMM 1856
Sect. I. *Erythrocistus* Dunal	Genus *Ladanium* Spach	Subgen. I. *Erythrocistus* Dunal
C. albidus L.	*L. officinarum* Spach *(C. ladanifer* L.)	Sect. I. *Macrostylia* Willk.
C. candidissimus Dunal *(C. ochreatus* C. Sm. ex Buch*)*	*L. laurifolium* Spach *(C. laurifolius* L.*)*	*C. vaginatus* Aiton *(C. symphytifolius* Lam.*)*
C. complicatus Lam. *(C. parviflorus* Lam.*)*	*L. cyprium* Spach *(C. ladanifer x C. laurifolius)*	*C. candidissimus* Dunal *(C. ochreatus* C. Sm. ex Buch*)*
C. creticus L.		Sect. II. *Brachystylia* Willk.
C. crispus L.	Genus *Rhodocistus* Spach	*C. albidus* L.
C. cymosus Dunal *(C. parviflorus x C. creticus)*	*R. berthelotianus* Spach *(C. symphytifolius* Lam.)	*C. polymorphus* Willk. *(C. creticus* L.*)*
C. heterophyllus Desf.		*C. creticus* L.
C. hybridus Vahl (?)	Genus *Cistus* (Tourn.) Spach	*C. crispus* L.
C. incanus L. *(C. albidus x C. crispus)*	Sect. I. *Rhodopsis* Spach	*C. heterophyllus* Desf.
C. parviflorus Lam.	*C. purpureus* Lam. *(C. ladanifer x C. creticus)*	*C. purpureus* Lam. *(C. ladanifer x C. creticus)*
C. purpureus Lam. *(C. ladanifer x C. creticus)*	Sect. II. *Eucistus* Spach	Sect. III. *Astylia* Willk.
C. sericeus Vahl *(C. albidus ?)*	*C. vulgaris* Spach *(C. creticus* L.)	*C. parviflorus* Lam.
C. undulatus Dunal *(C. creticus* L.*)*	Sect.III. *Ledonella* Spach	
C. vaginatus Dryand. *(C. symphytifolius* Lam.*)*	*C. parviflorus* Spach *(C. parviflorus* Lam.*)*	Subgen. II. *Leucocistus* Willk.
C. villosus Lam. *(C. creticus* L.*)*		Sect. IV. Stephanocarpus Spach
	Genus *Stephanocarpus* Spach	*C. monspeliensis* L.
Sect. II. *Ledonia* Dunal	*S. monspeliensis* Spach *(C. monspeliensis* L.*)*	*C. pouzolzii* Delile
C. clusii Dunal		*C. florentinus* Lam. *(C. monspeliensis x C. salviifolius)*
C. corbariensis Pourr. (*C. populifolius x C. salviifolius*)	Genus *Ledonia* Spach	Sect. V. *Ledonia* Spach
C. cyprius Lam. *(C. ladanifer x C. laurifolius)*	*L. heterophylla* Spach *(C. monspeliensis x C. populifolius)*	*C. ledon* Lam. *(C. laurifolius x C. monspeliensis)*
C. florentinus Lam. *(C. monspeliensis x C. salviifolius)*	*L. populifolia* Spach *(C. populifolius* L.*)*	*C. populifolius* L.
C. hirsutus Lam. *(C. psilosepalus* Sweet*)*	*L. hirsuta* Spach *(C. psilosepalus* Sweet*)*	*C. longifolius* Lam. *(C. monspeliensis x C. populifolius)*
C. ladaniferus L. *(C. ladanifer* L.*)*	*L. peduncularis* Spach *(C. salviifolius* L.*)*	*C. obtusifolius* Sweet *(C. psilosepalus x C. salviifolius)*
C. laurifolius L.		*C. hirsutus* Lam. *(C. psilosepalus* Sweet)
C. laxus Aiton *(C. populifolius x C. psilosepalus ?)*		*C. salviifolius* L.
C. ledon Lam. *(C. laurifolius x C. monspeliensis)*		Sect. VI. *Ladanium* Spach
C. longifolius Lam. *(C. monspeliensis x C. populifolius)*		*C. cyprius* Lam. *(C. ladanifer x C. laurifolius)*
C. monspeliensis L.		*C. ladaniferus* L. *(C. ladanifer* L.*)*
C. populifolius L.		*C. laurifolius* L.
C. salviifolius L.		Sect. VII. *Halimioides* Willk.
		C. clusii Dun.
		C. bourgaeanus Coss. *(C. libanotis* L.*)*
		C. sericeus Munbyi *(C. munbyi* Pomel*)*

Table 1. *(Continued)*

GROSSER 1903	DANSEREAU 1939	DEMOLY AND MONTSERRAT 1993 (Iberian species)
Group A.	Subgen. I. *Erythrocistus* (Dunal) Willk.	Subgen. I. *Cistus* L.
Sect. I. *Rhodocistus* (Spach) Grosser	Sect. I. *Macrostylia* Willk.	*C. albidus* L.
C. ochreatus C. Sm. ex Buch	*C. osbeckiaefolius* Webb ex Christ (*C. osbeckiifolius* Webb ex Christ)	*C. creticus* L.
C. symphytifolius Lam.	*C. symphytifolius* Lam.	*C. crispus* L.
Sect. II. *Eucistus* Spach	Sect. II. *Erythrocistus* Dunal	*C. heterophyllus* Desf.
C. albidus L.	*C. albidus* L.	
C. villosus L. *(C. creticus* L.*)*	*C. villosus* L. *(C. creticus* L.*)*	Subgen. II. *Leucocistus* Willk.
C. crispus L.	*C. crispus* L.	Sect. 1. *Ledonia* Dunal
C. heterophyllus Desf.	*C. heterophyllus* Desf.	*C. monspeliensis* L.
Sect. III. *Ledonella* Spach	Sect. III. *Ledonella* Spach	*C. populifolius* L.
C. parviflorus Lam.	*C. parviflorus* Lam.	*C. psilosepalus* Sweet
		C. salviifolius L.
Group B.	Subgen. II. *Leucocistus* Willk.	Sect. 2. *Ladanium* (Spach) Gren.
Sect. IV. *Stephanocarpus* (Spach) Willk.	Sect. IV. Stephanocarpoidea Rouy et Foucaud	*C. ladanifer* L.
C. monspeliensis L.	*C. varius* Pourr. *(C. pouzolzii* Del.*)*	*C. laurifolius* L.
Sect. V. *Ledonia* Dunal	Sect. V. *Stephanocarpus* (Spach) Gren.	
C. populifolius L.	*C. monspeliensis* L.	Subgen. III. *Halimioides* (Willk.) Demoly & P. Monts.
C. hirsutus Lam. *(C. psilosepalus* Sweet*)*	Sect. VI. *Ledonia* Dunal	*C. clusii* Dunal
C. salviifolius L.	*C. populifolius* L.	*C. libanotis* L.
	C. hirsutus Lam. *(C. psilosepalus* Sweet)	
Group C.	*C. salviifolius* L.	
Sect. VI. *Ladanium* (Spach) Willk.	Sect. VII. *Ladanium* (Spach) Gren. et Godr.	
C. ladaniferus L. *(C. ladanifer* L.*)*	*C. ladaniferus* L. *(C. ladanifer* L.*)*	
C. laurifolius L.	*C. laurifolius* L.	
Sect. VII. *Halimioides* Willk.	Sect. VIII. *Halimioides* Willk.	
C. rosmarinifolius Pourr. *(C. clusii* Dunal*)*	*C. libanotis* L. *(C. clusii* Dunal)	
C. bourgaeanus Coss. *(C. libanotis* L.*)*	*C. bourgeanus* Coss. *(C. libanotis* L.)	
C. sericeus Munby *(C. munbyi* Pomel)	*C. munbyi* Pomel	

Taxa in brackets as interpreted

species described more recently, the genus is currently thought to comprise approximately 20 species, of which 16 occur in Europe (Warburg, 1968), 11 in Spain (Martín and Guinea, 1949), 12 in Iberia (Demoly and Montserrat, 1993), and 12 in Morocco (Soriano, 2002) (Figure 1). The highest species diversity therefore occurs in the western Mediterranean, where 14 species are distributed in the Iberian Peninsula and Morocco.

Disparate infrageneric classifications of *Cistus* have been proposed (Table 1). In the last taxonomic treatment three subgenera, namely *Cistus*, *Leucocistus*, and *Halimioides*, are described based on morphological characters (Demoly and Montserrat, 1993). The subgenus *Halimioides* (three species) is distributed exclusively in the western Mediterranean, whilst the subgenera *Leucocistus* (eight species) and *Cistus* (nine species) occur in the Mediterranean basin and the Canary Islands. This widespread distribution of *Cistus* subgenera and species clearly indicates the successful mobility of seeds and colonization in Mediterranean habitats.

Evolutionary mechanisms responsible for the morphological diversity within *Cistus* remain poorly understood. Plants are predominantly self-incompatible (Bosch, 1992) promoting crossing between individuals of the same and different species. Identification in the field of individuals as hybrids is relatively easy because they display characteristics that are intermediate between those of nearby, putative progenitors. Crossing between two plants of any species potentially generates offspring with intermediate traits, particularly when they are closely related congeners (Demoly, 1996). Hybrid polyploidy (allopolyploidy) has not played an important role in speciation of *Cistus*, as all species display a chromosome number of 2n = 18. In fact, variation of DNA content is not significant among species (Ellul et al., 2002).

A proper phylogeny of *Cistus* has not been proposed to date. Dansereau (1939) outlined a phyletic diagram based on morphological features. Examination of 13 isozyme loci indicates high values of genetic divergence among four Canarian species (Batista et al., 2001), although evolutionary relationships of island endemics with

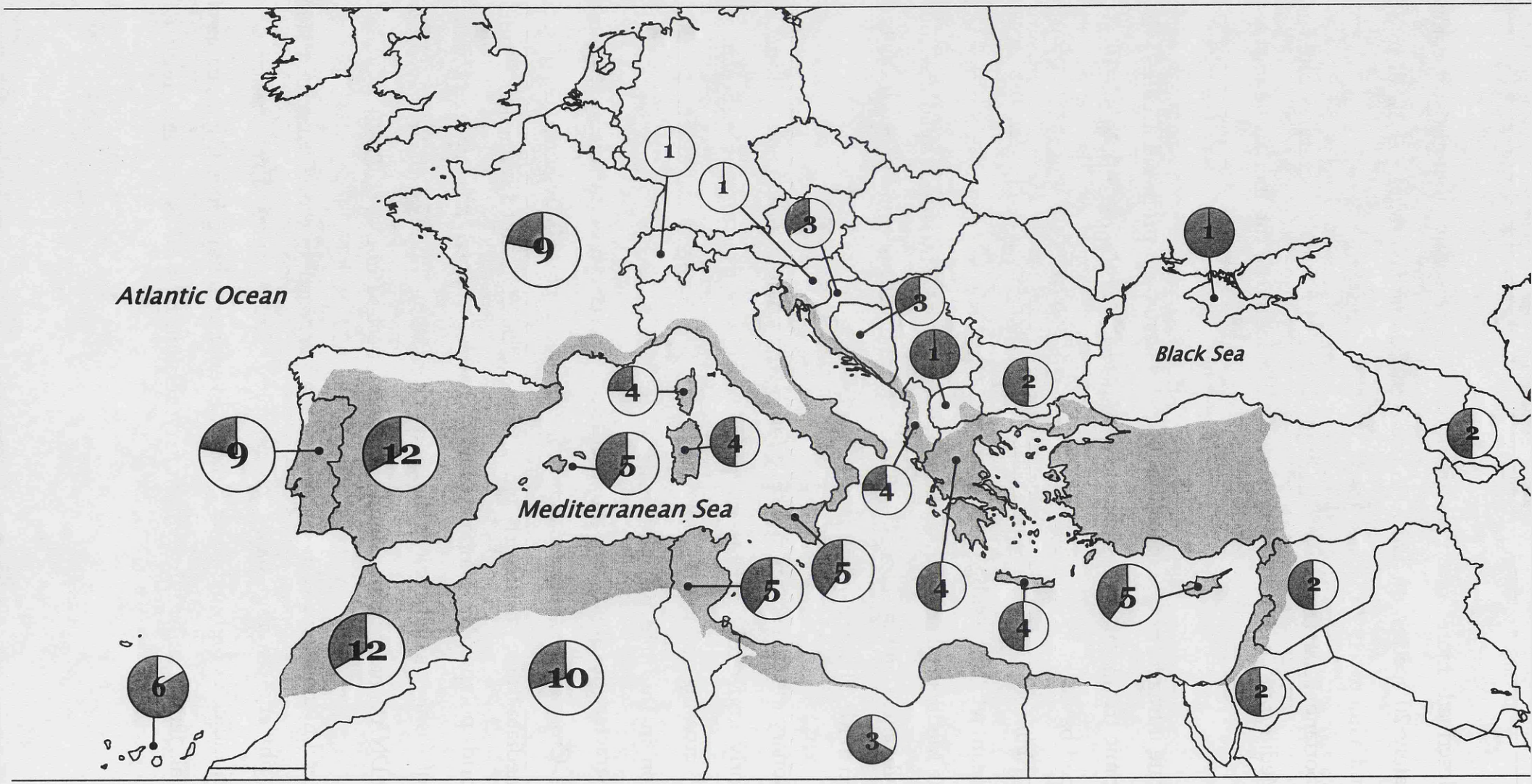

Fig. 1. Distribution map and number of *Cistus* species. Pie diagrams include proportion of white-flowered (white) and purple-flowered (grey) species in country. Notice the highest species diversity in the western Mediterranean. The Mediterranean region shown in grey.

respect to continental species remain unknown. Phylogenetic relationships among Cistaceae genera indicate that *Cistus* is closely related to *Halimium* and *Helianthemum* (Savolainen et al., 2000; Arrington and Kubitzki, 2003). In addition, angiosperm phylogenies reveal that the family forms a lineage coupled with Dipterocarpaceae and Sarcolaenaceae (Soltis et al., 2000). A larger sample is needed however to determine sister-group relationships of Cistaceae and *Cistus*.

Four basic objectives are addressed in the present study: (1) to evaluate congruence between nuclear (ITS) and plastid (*trnL-trnF*, *trnK-matK*) sequences; (2) to identify major lineages and test the monophyly of infrageneric groupings recognized in existing classifications of *Cistus;* (3) to interpret evolution of key morphological characters; (4) to describe biogeographic patterns in the Mediterranean basin and in the colonization of the Canary Islands.

2. Materials and methods

2.1. DNA extraction, gene amplification and sequencing

A total of 47 individuals representing the 20 species of *Cistus*, one of *Fumana*, two of *Halimium*, two of *Helianthemum*, and one of *Tuberaria* were sampled for ITS, *trnL-F* and *trnK-matK* sequencing (Appendix 1). Total genomic DNA was extracted from material collected in the field, material in the living collections of R. Page, O. Filippi, and the Royal Botanic Garden of Madrid, and from two herbarium specimens (MA). Field collections were dried in silica gel. DNA was extracted using Kneasy Plant Mini Kit (QIAGEN Inc., California) and amplified using the PCR (Polymerase Chain Reaction) on a Perkin-Elmer PCR System 9700 (California) or an MJ Research (Massachusetts) thermal cycler. After 1-4 min pretreatment at 94 °C, PCR conditions were: 24-35 cycles of 1 min at 94 °C, 30 s -1 min at 50-52 °C, and 1-2 min at 72 °C. Standard primers were used for amplification of the *trnL*(UAA)-*trnF*(GAA) spacer (Taberlet et al., 1991), the *trnK-matK* spacer (*trnK*-3914F, *matK*-1470R; Johnson and Soltis, 1994), and the ITS region (Sun et al., 1994 for 17SE; White et al., 1990 for ITS4). A volume of 1 µL of dimethyl-sulfoxide (DMSO) was included in each 25 µl reaction. Amplified products were

cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v.2.0, Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism model 3700 automated sequencer. PCR primers were used for cycle sequencing of the *trnL-F* and the *trnK-matK* spacers, while the ITS5 and ITS4 (Sun et al., 1994) primers were used for cycle sequencing the ITS region. Sequenced data were assembled and edited using the program Seqed (Applied Biosystems, California). The limits of the regions were determined by position of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

2.2 Molecular analysis

Phylogenetic analyses were performed on three molecular datasets (*trnL-F*, *trnK-matK*, ITS) using the same methodology and a similar number of sequences from each of the 20 species of *Cistus* and six of Cistaceae (Appendix 1). In addition, an analysis of *trnL-F* sequences from Old World Cistaceae (*Fumana, Helianthemum, Tuberaria, Halimium, Cistus*) and Dipterocarpaceae was performed to investigate relationships of *Cistus* with respect to another Cistaceae. In this analysis, four Dipterocarpaceae (*Dipterocarpus, Parashorea, Shorea, Hopea*) from GenBank (Li et al., unpublished) were used as outgroup taxa on the basis of an earlier *rbcL* phylogeny (Ducousso et al., 2004).

Sequences were aligned using Clustal X 1.62b (Thompson et al., 1997), with further adjustments by visual inspection. Insertion/deletion mutations (indels) were manually coded for parsimony analyses as appended characters following the logic of Kelchner (2000) and Simmons and Ochoterena (2000). Maximum Parsimony (MP) and Bayesian Inference (BI) analyses were then performed on each dataset. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Heuristic searches were replicated 100 times with random taxon-addition sequences, Tree-

Bisection-Reconnection (TBR) branch swapping, and the options MulTrees and Steepest Descent in effect. Additionally, as a result of memory limitation in completing the analysis of *trnL-F* sequences of the Dipterocarpaceae-Cistaceae matrix, ten trees only were saved from each of the 1000 replicates to minimize time searching thousands of trees. All trees thus collected were combined and used as starting trees, with MulTrees on and no tree limit (these trees were then swapped to completition) and Subtree-Pruning-Regrafting (SPR) (Salamin et al., 2003). Internal support was assessed using 1000 replicates with simple taxon addition and SPR branch swapping, but permitting only ten trees per replicate to be held (Chase et al., 2003).

To determine the simplest model of sequence evolution that best fits the sequence data the Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) were implemented using MrModeltest 1.1b (Nylander, 2002). A Bayesian Inference analysis was conducted on each dataset using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) and sampling for one million generations (four MCMC, chain temperature = 0.2; sample frequency = 100; burn-in < 500). A 50% majority-rule consensus tree was calculated for each matrix from the pooled sample using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro et al., 2003).

To assess whether data provide significantly less support for a specified alternative topology, we used the Significantly Less Parsimonious test of Templeton (SLP_T) (Templeton, 1983) and compared matrices and most parsimonious topologies recovered by analyzing ITS, *trnL-F*, and *trnK-matK* sequences. SLP_T was implemented in PAUP* using the strict consensus tree obtained from parsimony analyses of the data (Johnson and Soltis, 1998).

2.3 Morphological characters

The distribution of ten morphological characters, upon which classification of *Cistus* has been traditionally based, is indicated in Appendix 2. Information on some characters is missing for some species and we consequently performed reconstructions of two

vegetative (shape and base of leaves) and four reproductive (sepal and fruit-valve number, petal color, style length) characters. Patterns of evolution were explored using the character-state optimization function of MacClade 4.06 (Maddison and Maddison, 1992), assuming Fitch parsimony. Both ACTRAN (maximizing the proportion of the homoplasy that is accounted by parallelism) and DELTRAN (maximizing the proportion accounted for by reversals) optimizations were considered and analyzed. Characters were traced initially onto the strict consensus of shortest trees obtained. To gain further insights into morphological character evolution, the MP tree displaying most congruence with the BI tree, under the simplest model of sequence evolution, was chosen (see below).

3. Results

3.1. Characteristics of trnL-F, *ITS, and trnK-*matK *sequences*

The characteristics of the three datasets are summarized in Table 2. Within *Cistus, trnL-F* sequence divergence ranges from 0.0% (between the 17 conspecific accessions and between *C. clusii-C. munbyi, C. symphytifolius-C. chinamadensis, C. albidus-C. creticus*) to 3.15% (between *C. parviflorus* 1-*C. monspeliensis* 1) using the K-2-p model of evolution; *trnK-matK* sequence divergence ranges from 0.0% (between 12 conspecific accessions and between *C. albidus-C. creticus, C. albidus-C. heterophyllus, C. creticus-C. heterophyllus*) and 1.78% (between *C. salviifolius-C. osbeckiifolius*); and ITS sequence divergence ranges from 0.0% (between eight conspecific accessions) to 4.86% (between *C. crispus-C. parviflorus*). Nucleotide additivity for direct ITS sequencing was clearly observed in direct and reverse chromatograms at 15 positions of 10 accessions (see Appendix 2). More than one ITS copy with different sequence length was also detected in two accessions (*C. psilosepalus* 1, *C. parviflorus* 1). A single gap allowed re-establishing nucleotide chromatogram matching, and the resulting sequence was used in the phylogenetic analyses.

Table 2. Summary of phylogenetic characteristics obtained from the analyses of ITS, *trnL-trnF*, and *trnK-matK* sequences of the Cistaceae and *Cistus*

	ITS				*trnL-trnF*	*trnK-matK*
	ITS region	ITS-1	5.8 S	ITS-2		
Cistaceae						
Length range (bp)	567-696	202-275	166-167	199-254	377-461	1302-1357
Aligned length (bp)	698	274	168	256	505	1403
Number of variables/informative characters	203/104	108/62	4/3	91/39	127/66	265/143
Maximum sequence divergence (K-2-p)	20.03%	29.70%	2.47%	33.86%	20.07%	13.75%
Informative indels (no. bp)	19(1-41)	9(1-41)	0	10(1-29)	15(1-26)	17(1-48)
CI' (CI)	0.64 (0.78)	-	-	-	0.84 (0.9)	0.87 (0.92)
RI	0.82	-	-	-	0.93	0.95
Mean G+C content	65%	69%	49%	68%	33%	33%
Cistus						
Number of variables/informative characters	92/73	58/45	1/0	33/28	48/42	56/46
Maximum sequence divergence (K-2-p)	4.86%	9.33%	0.60%	5.26%	3.15%	1.78%
Informative indels (no. bp)	7(1-2)	4(1-2)	0	3(1-2)	10(1-26)	3(1-48)
Number of nucleotide additivities	15	10	0	5	0	0
Number of accessions with nucleotide additivities	10	7	0	7	0	0

3.2. Phylogenetic analyses

Availability (Li et al., in GenBank) and alignability (clustal X, Thompson et al., 1997) of *trnL-F* sequences from four Dipterocarpaceae genera (*Dipterocarpus, Parashorea, Shorea, Hopea*) allowed performing suitable phylogenetic analysis of Cistaceae-Dipterocarpaceae accessions. MP and BI analyses recognize Cistaceae as monophyletic, with 100% bootstrap value (BS) and 100 posterior probability (PP). The strict consensus tree of 362,200 MP trees is shown in Fig. 2. Within Cistaceae, a successive branching is depicted in the strict consensus tree, in which *Fumana* comes out first (92% BS) followed by *Helianthemum* (100% BS), and then *Tuberaria*. Accessions of *Halimium* and *Cistus* form a largely unresolved, monophyletic group (81% BS). Bayesian inference, using GTR+G as the simplest model of sequence evolution, reached equilibrium after 350,000 generations. The BI reconstruction is mostly consistent with the strict consensus tree, but more resolved: (i) *Halimium calycinum* is sister to *Cistus* (76 PP), whilst *Halimium umbellatum* is nested within a group of white-flowered *Cistus* species (67 PP); (ii) within this group, a subgroup of six species (*C. laurifolius, C. parviflorus, C. psilosepalus, C. pouzolzii, C. populifolius, C. monspeliensis*) is also retrieved (80 PP); (iii) *C. monspeliensis* and *C. populifolius* are sister species (95 PP) (results not shown). In the MP and BI analyses, accessions of the same species either formed monophyletic groups or were placed in unresolved polytomies. We used hereafter *Fumana thymifolia* as the outgroup taxon based on its sister-group relationship to the rest of Cistaceae in the *trnL-F* (Fig. 2) and *rbcL* (Guzmán et al., unpublished) analyses. The analysis of Cistaceae-only accessions using *Fumana* as the outgroup taxon resulted in a similar resolution, support, and consistency indices (see Table 2). The GTR+G model was also selected for the *trnK-matK* data set. MP and BI analyses yielded similar topology, although with less resolution and lower support values in the MP (results not shown). The strict consensus tree of the combined *trnK-matK* and *trnL-F* sequences depicts *Halimium* and *Cistus* as monophyletic (90% BS), and a basal polytomy is formed by *Halimium umbellatum* and two *Cistus* clades (Fig. 3A). A well-resolved *Cistus* clade (clade 1) comprises all purple-flowered species except for *C. parviflorus* (99% BS). A second *Cistus* clade (clade 2) consists of all white-flowered species plus *C. parviflorus* (98% BS). Bayesian Inference

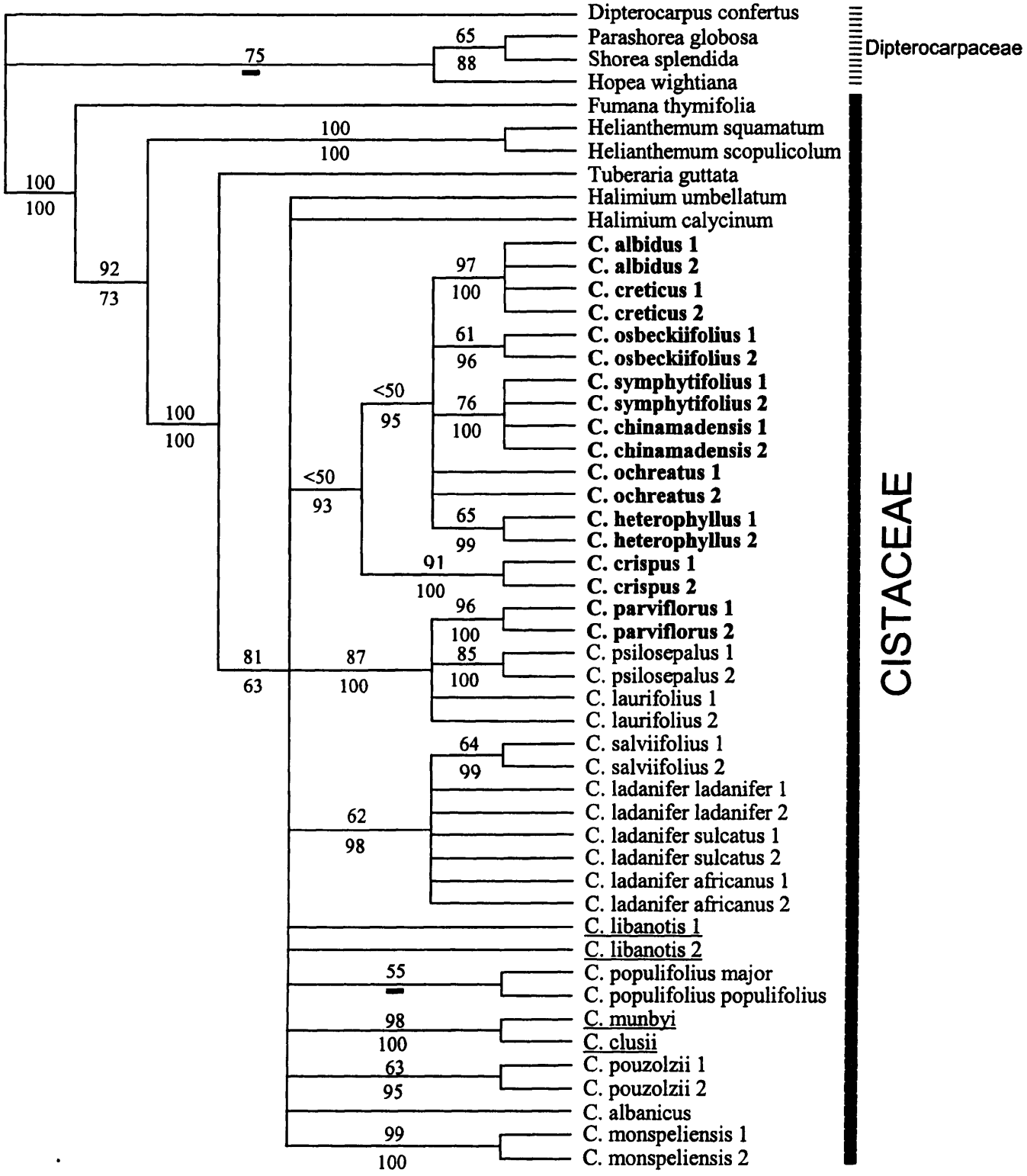

Fig. 2. Strict consensus tree of 362,200 shortest trees of 220 steps (CI = 0.90; CI′ = 0.83, excluding uninformative characters; RI = 0.94) from the analysis of *trnL-F* sequences. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR+G as the simplest model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen below branches (–). Taxa circumscription in subgenera is coded as followed: *Cistus* (in bold); *Leucocistus* (in roman); and *Halimioides* (underlined).

analysis of the combined *trnK-matK* and *trnL-F* matrix using the common, simplest model of sequence evolution for both data sets (GTR+G) reached equilibrium after 40,000 generations. Again, the BI analysis displays better resolution and higher support values than those of the MP analysis, including the sister relationship of *Halimium calycinum* to a group of all *Cistus* species and *Halimium umbellatum* and of this *Halimium* species to the group of white-flowered *Cistus* species (clade 2). High BS and PP values (over 85 support values) were retrieved for 11 groups of conspecific accessions (Fig. 3A).

The analysis of ITS sequences yielded limited resolution (Fig. 3B). Eight conspecific accessions are resolved into well-defined monophyletic groups, mostly in agreement with those in the plastid DNA tree (Fig. 3A). In addition, other supported clades are: the four accessions of *C. albidus-C. creticus* (91% BS); the six Canarian accessions (73% BS); and the two accessions of *C. clusii-C. munbyi* (87% BS). Bayesian inference using the selected GTR+*G*+*I* model reached equilibrium after 50,000 generations. The BI analysis retrieved similar relationships to those in Fig. 3B, plus a group of *C. psilosepalus* sister to *C. ladanifer* accessions (88 PP) and *C. heterophyllus* sister to the Canarian group (82 PP). Visual inspection of ITS chromatograms revealed 13 positions containing nucleotide double-peaks (Table 2). Although it was not possible to determine whether, in some cases, double-peak patterns may be the result of sequencing artifacts, equimolar proportions of alternative nucleotide peaks in many accessions suggested the presence

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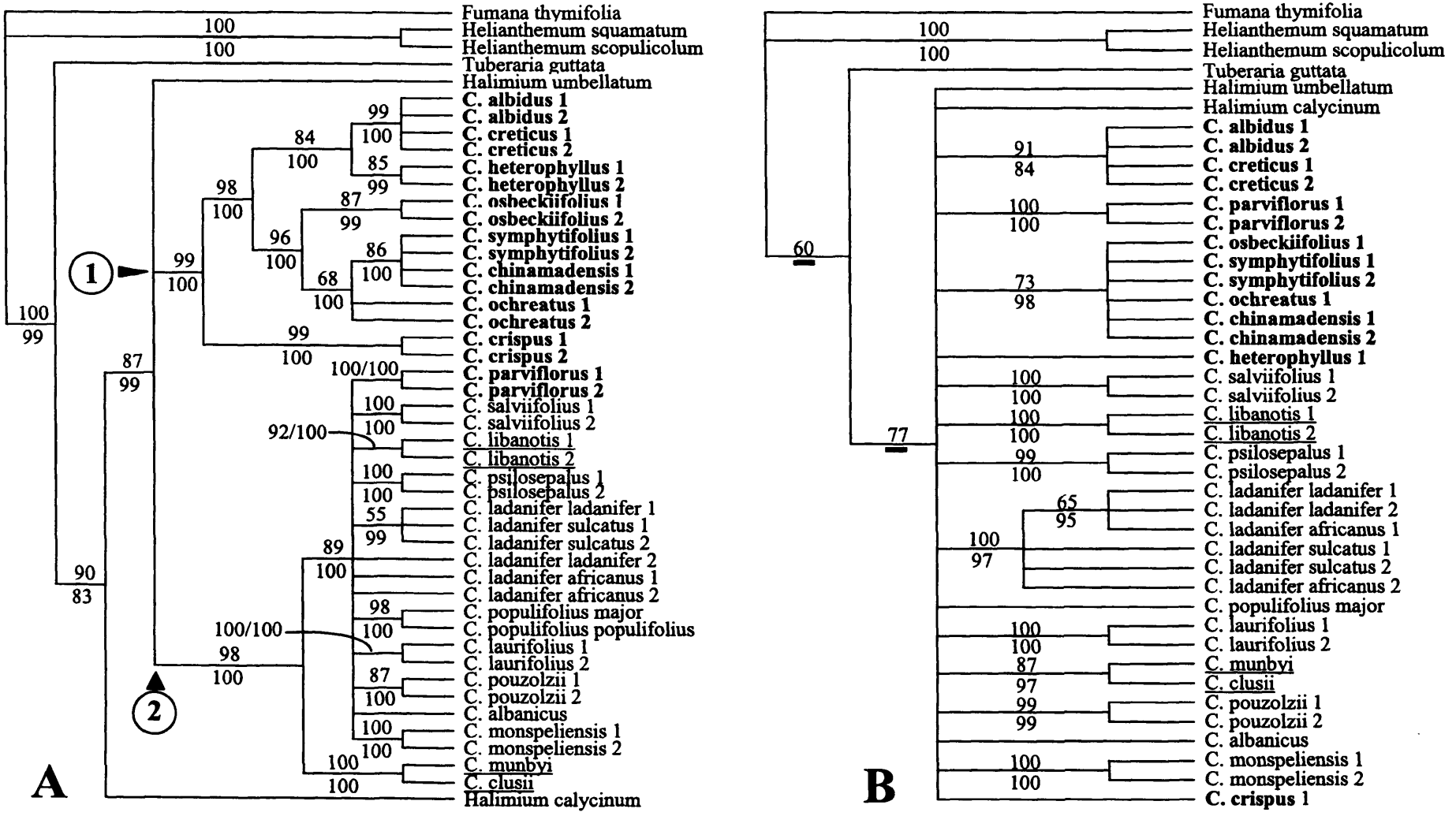
Fig. 3. Strict consensus trees of MP analyses. *Fumana thymifolia* served as the outgroup taxon. Insertions/deletions (indels) recorded as additional characters. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR+G model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen below branches (–). Two major clades indicated in circled numbers. (A) Strict consensus tree of 224 shortest trees of 512 steps (CI = 0.90; CI′ = 0.85 excluding uninformative characters; RI = 0.94) from the combined analysis of *trnL-F* and *trnK-matK* sequences. (B) Strict consensus tree of 69,486 most parsimonious trees of 933 steps (CI = 0.78; CI′ = 0.64; RI = 0.82) from the analysis of ITS-region sequences. Taxa circumscription in subgenera is coded as followed: Cistus (in bold); Leucocistus (in roman); and Halimioides (underlined).


A
Fumana thymifolia
Helianthemum squamatum
Helianthemum scopulicolum
Tuberaria guttata
Halimium umbellatum
C. albidus 1
C. albidus 2
C. creticus 1
C. creticus 2
C. heterophyllus 1
C. heterophyllus 2
C. osbeckiifolius 1
C. osbeckiifolius 2
C. symphytifolius 1
C. symphytifolius 2
C. chinamadensis 1
C. chinamadensis 2
C. ochreatus 1
C. ochreatus 2
C. crispus 1
C. crispus 2
C. parviflorus 1
C. parviflorus 2
C. salviifolius 1
C. salviifolius 2
C. libanotis 1
C. libanotis 2
C. psilosepalus 1
C. psilosepalus 2
C. ladanifer ladanifer 1
C. ladanifer sulcatus 1
C. ladanifer sulcatus 2
C. ladanifer ladanifer 2
C. ladanifer africanus 1
C. ladanifer africanus 2
C. populifolius major
C. populifolius populifolius
C. laurifolius 1
C. laurifolius 2
C. pouzolzii 1
C. pouzolzii 2
C. albanicus
C. monspeliensis 1
C. monspeliensis 2
C. munbyi
C. clusii
Halimium calycinum
1
2
B
Fumana thymifolia
Helianthemum squamatum
Helianthemum scopulicolum
Tuberaria guttata
Halimium umbellatum
Halimium calycinum
C. albidus 1
C. albidus 2
C. creticus 1
C. creticus 2
C. parviflorus 1
C. parviflorus 2
C. osbeckiifolius 1
C. symphytifolius 1
C. symphytifolius 2
C. ochreatus 1
C. chinamadensis 1
C. chinamadensis 2
C. heterophyllus 1
C. salviifolius 1
C. salviifolius 2
C. libanotis 1
C. libanotis 2
C. psilosepalus 1
C. psilosepalus 2
C. ladanifer ladanifer 1
C. ladanifer ladanifer 2
C. ladanifer africanus 1
C. ladanifer sulcatus 1
C. ladanifer sulcatus 2
C. ladanifer africanus 2
C. populifolius major
C. laurifolius 1
C. laurifolius 2
C. munbyi
C. clusii
C. pouzolzii 1
C. pouzolzii 2
C. albanicus
C. monspeliensis 1
C. monspeliensis 2
C. crispus 1

of more than one ITS copies. This view is supported by the facts that forward and reverse chromatograms displayed double peaks of the same nucleotide proportions and that five affected matrix positions turned to be parsimony-informative characters.

In the analysis of Cistaceae, resolution and support at clade tips and deep nodes is higher in plastid than in nuclear trees. Consensus-tree topologies display polytomies primarily as a result of insufficient number of informative characters and character incongruence across accessions. In *Cistus*, the number of parsimony-informative characters is higher in the ITS (73) than in the *trnL-F* (42) and *trnK-matK* (46) sequences, indicating that the ITS analysis had a sufficient number of informative characters for better resolution. A search for the causes behind low levels of resolution revealed higher measure of fit for the *trnL-F* and *trnK-matK* analyses (CI′ = 0.84 and CI′ = 0.87, respectively) than that for the ITS analysis (0.64). These values fall into the CI and RI range provided by Álvarez and Wendel (2003), who also reported that ITS datasets have higher levels of homoplasy in angiosperms than plastid datasets. Additionally, the occurrence of more than one nucleotide (additivity) at the same seven informative sites in some ITS accessions contributed to a low resolution, as a result of multiple searches using alternative character states.

3.3. Data congruence and combined phylogeny

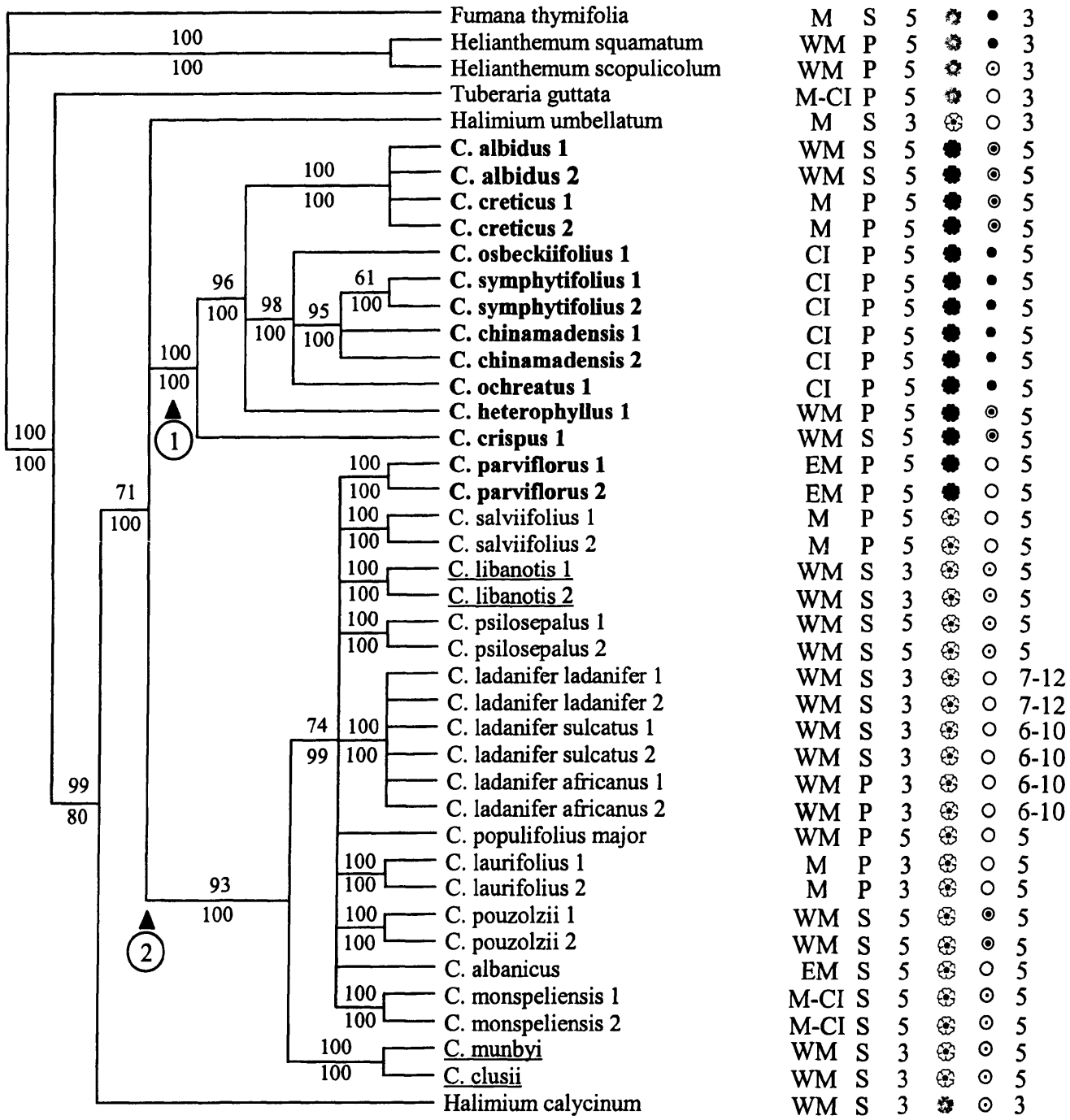
The plastid genome is generally considered free from recombination and the *trnL-F* and *trnK-matK* sequences consequently share a hypothetical common phylogenetic history. This provides a strong argument for inferring character evolution by combining *a priori* both data sets. Additionally, a significance test for heterogeneity between nuclear and plastid data sets was implemented. Characters in each of the three data sets statistically support alternative topologies found in the set of the shortest trees recovered for those data sets (Table 3). As statistical sub-optimality exists in at least one direction in each comparison, the SLP_T supports data homogeneity (Johnson and Soltis, 1998). The combined plastid and nuclear data matrix of 42 samples consisted of 2606 characters, of

Table 3. Results of the Templeton's test (SLP_T) on alternative topologies of strict consensus trees

Data set	Alternative topology	Increase	Decrease	Net	Probability
ITS	*trn*L-F	28	8	48	0.028*
*trn*L-F	ITS	34	2	36	0.0001*
ITS	*trnK-mat*K	27	4	50	0.825
*trnK-mat*K	ITS	58	49	67	0.0001*
*trn*L-F	*trnK-mat*K	18	13	23	0.24
*trnK-mat*K	*trn*L-F	19	1	28	0.0021*

Probability values greater than 0.05 indicate that the alternative topology is not significantly less parsimonious than at least one shortest tree.

which the number of variable/parsimony informative characters was 595/313 in the Cistaceae (Table 2). The strict consensus tree reveals, once again, a well-defined assemblage of all the *Halimium* and *Cistus* accessions (99% BS), in which *Halimium calycinum* is sister (71% BS) to a group formed by *Halimium umbellatum* and two clades of *Cistus* (Fig. 4). Clade 1 contains exclusively purple-flowered, 5-sepaled, mid-to-long styled species (subgenus Cistus) (100% BS). Within clade 1, *C. crispus* is sister (96% BS) to the remaining members; they, in turn, form three subclades. The first is a well-defined (100% BS) group of *C. albidus* and *C. creticus* accessions. The second forms a well-supported group (98% BS) of Canarian species, and includes a subgroup of *C. symphytifolius* and *C. chinamadensis* accessions (95% BS). *Cistus heterophyllus* constitutes the third, unresolved subclade. Clade 2 contains all white-flowered species of subgenera Leucocistus and Halimioides, plus the pinkish-flowered *C. parviflorus* (93% BS). The three species of subgenus Halimioides do not form a monophyletic group. Whilst *C. munbyi* and *C. clusii* constitutes a clade (100% BS) that is resolved as sister to the rest of species in clade 2, *C. libanotis* is unresolved in the large polytomy of white-flowered species. The results from the BI analysis, implementing partitions with the respective simplest models of evolution, were consistent with the strict consensus of the MP trees, but with better resolution and similar or higher support values in most cases. Interestingly, a group of white-flowered *Cistus-Halimium* species is resolved in the BI tree (96 PP), displaying a pectinate topology and high support values (results not shown). In this BI tree, *Halimium umbellatum* is sister to the white-flowered species of


Distribution
Leaf base
No. sepals
Petal color
Style length
No. valves
Fumana thymifolia M S 5 3
Helianthemum squamatum WM P 5 3
Helianthemum scopulicolum WM P 5 3
Tuberaria guttata M-CI P 5 3
Halimium umbellatum M S 3 3
C. albidus 1 WM S 5 5
C. albidus 2 WM S 5 5
C. creticus 1 M P 5 5
C. creticus 2 M P 5 5
C. osbeckiifolius 1 CI P 5 5
C. symphytifolius 1 CI P 5 5
C. symphytifolius 2 CI P 5 5
C. chinamadensis 1 CI P 5 5
C. chinamadensis 2 CI P 5 5
C. ochreatus 1 CI P 5 5
C. heterophyllus 1 WM P 5 5
C. crispus 1 WM S 5 5
C. parviflorus 1 EM P 5 5
C. parviflorus 2 EM P 5 5
C. salviifolius 1 M P 5 5
C. salviifolius 2 M P 5 5
C. libanotis 1 WM S 3 5
C. libanotis 2 WM S 3 5
C. psilosepalus 1 WM S 5 5
C. psilosepalus 2 WM S 5 5
C. ladanifer ladanifer 1 WM S 3 7-12
C. ladanifer ladanifer 2 WM S 3 7-12
C. ladanifer sulcatus 1 WM S 3 6-10
C. ladanifer sulcatus 2 WM S 3 6-10
C. ladanifer africanus 1 WM P 3 6-10
C. ladanifer africanus 2 WM P 3 6-10
C. populifolius major WM P 5 5
C. laurifolius 1 M P 3 5
C. laurifolius 2 M P 3 5
C. pouzolzii 1 WM S 5 5
C. pouzolzii 2 WM S 5 5
C. albanicus EM S 5 5
C. monspeliensis 1 M-CI S 5 5
C. monspeliensis 2 M-CI S 5 5
C. munbyi WM S 3 5
C. clusii WM S 3 5
Halimium calycinum WM S 3 3
100
100
100
100
96
100
98
100
95
100
61
100
100
100
100
100
71
100
99
80
100
100
100
100
100
100
100
100
74
99
100
100
100
100
93
100
100
100
100
100
100
100
100
100
1
2

Cistus (100 PP), which are also arranged in a pectinate fashion with *C. munbyi-C. clusii* (99 PP) as the earliest diverging group, followed by *C. libanotis* (86 PP), *C. albanicus* (56 PP), and then a biphyletic group consisting of *C. salviifolius* sister to *C. ladanifer* (97 PP) and *C. monspeliensis* sister to the remaining five species (99 PP) (results not shown). Multiple conspecific accessions within clade 2 form well-supported monophyletic groups (100% BS, 100 PP) in eight cases (*C. parviflorus, C. salviifolius, C. libanotis, C. psilosepalus, C. ladanifer, C. laurifolius, C. pouzolzii, C. monspeliensis*).

3.4. Character-state reconstruction

A summary of significant character states obtained from the literature and from our own observations is shown in Figure 4. Exploration of character changes and ancestral state reconstruction was undertaken using the total-evidence analysis of nuclear and plastid sequences. ACCTRAN and DELTRAN optimizations gave extremely similar results, and only results using ACCTRAN are presented. The most likely of the shortest trees was chosen based on congruence with the BI analysis under the simplest model of sequence evolution (Fig. 5). MacClade reconstructions of character states indicate that leaf shape, sepal number, petal color, and style length are homoplastic in the *Cistus-Halimium* assemblage. For example, purple petals seem to have occurred twice, being the only flower color maintained in the eight species of clade 1. Trace of the four statesfor style length (sessile, shorter, similar, and longer than stamens) appears to be extremely complex, arising many times not only in this assemblage, but also in the

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Fig. 4. Strict consensus tree of 328 shortest trees of 895 steps (CI = 0.81; CI′ = 0.70; RI = 0.85) from the combined analysis of *trnL-F, trnK-matK,* and ITS sequences (total-evidence tree). Insertions/deletions (indels) recorded as additional characters. *Fumana thymifolia* served as the outgroup taxon. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities. Species distribution (M, Mediterranean; WM, western Mediterranean; EM, eastern Mediterranean; CI, Canary Islands) and five relevant morphological characters are plotted on the right side of the tree: leaf base (P, petiolate; S, sessile), number of sepals (3, 5); petal color (✿, yellow; ⊛, white; ●, purple); style length (O, sessile; ⊙, shorter than stamens; ◉, as long as stamens; •, longer than stamens); and number of fruit valves (3, 5, 6 or more). Taxa circumscription in subgenera is coded as followed: Cistus (in bold); Leucocistus (in roman); and Halimioides (underlined).

Cistaceae (Fig. 5A). The number of fruit valves is not a synapomorphy supporting the monophyly of *Cistus*, (which has five or more valves), in contrast to the three valves found in the remaining seven genera of Cistaceae. Remarkably, the three subspecies of *C. ladanifer* do not display five valves. Rather, between 6 and 12 fruit divisions are observed within this species, representing a unique increment in fruit segmentation from a 3-valved ancestor (Fig. 5B).

4. Discussion

4.1. Systematic implications

Analysis of *trnL-F* sequences supports the monophyly of Cistaceae genera (*Fumana, Helianthemum, Tuberaria, Halimium, Cistus*) (Fig. 2). All phylogenetic analyses are congruent with the monophyly of the *Cistus-Halimium* assemblage. A close relationship between these two genera was suggested in a phyletic diagram by Dansereau (1939). The two representatives of *Halimium* section Halimium (*H. umbellatum*) and section Commutata (*H. calycinum*) appear to have arisen from the same lineage involved in the formation of *Cistus* (Fig. 4). Although we obtained limited phylogenetic support sister-group relationship between *Halimium umbellatum* and the white-flowered species of *Cistus* is observed in some reconstructions (Fig. 5). In fact, the three species (*C. clusii, C. munbyi, C. libanotis*) of *Cistus* subgenus *Halimioides* are morphologically similar to *Halimium* in terms of leaf shape (linear), sepal number (3), and seed product (oligosperm placenta). *Cistus* subgenus *Halimioides* was recognized by some authors (Demoly and Montserrat, 1993) but not by others (Dunal, 1824; Willkomm, 1856; Dansereau, 1939). None of these taxonomic treatments of *Cistus* (Table 1) is fully congruent with the strict consensus tree of the combined analysis of *trnL-F, trnK-matK,* and ITS sequences (Fig. 5). The division of *Cistus* into two more subgenera formed by

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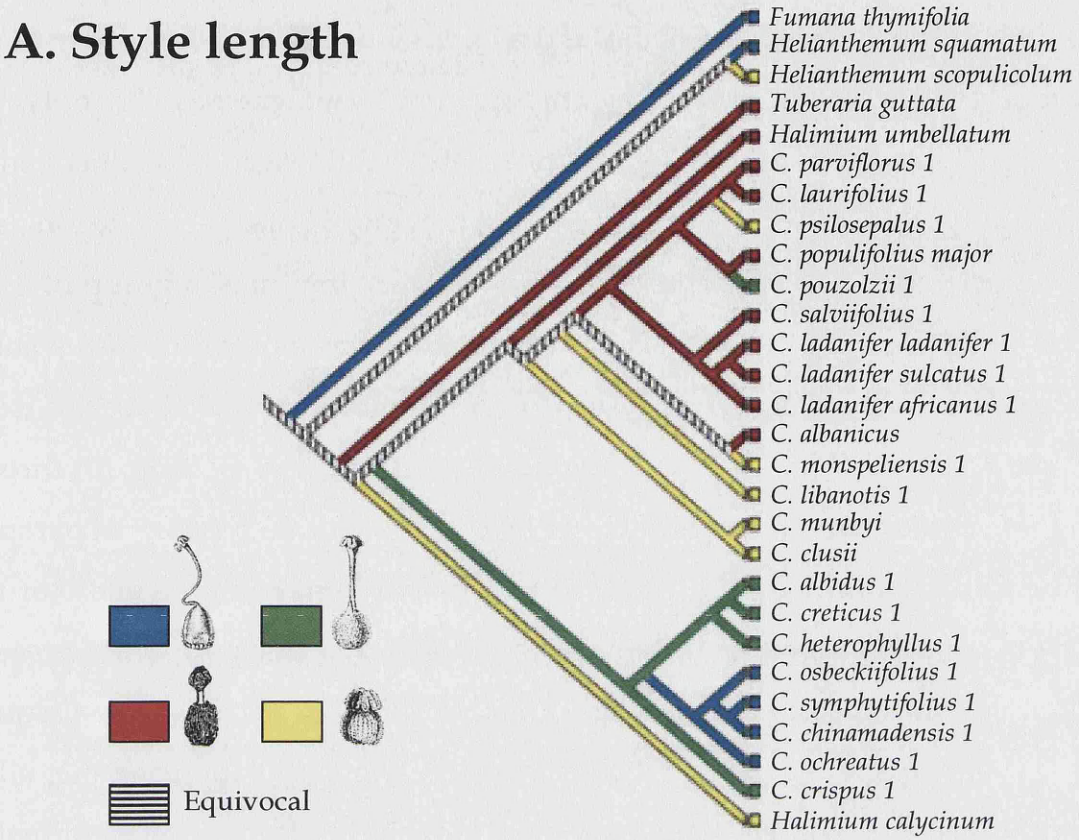
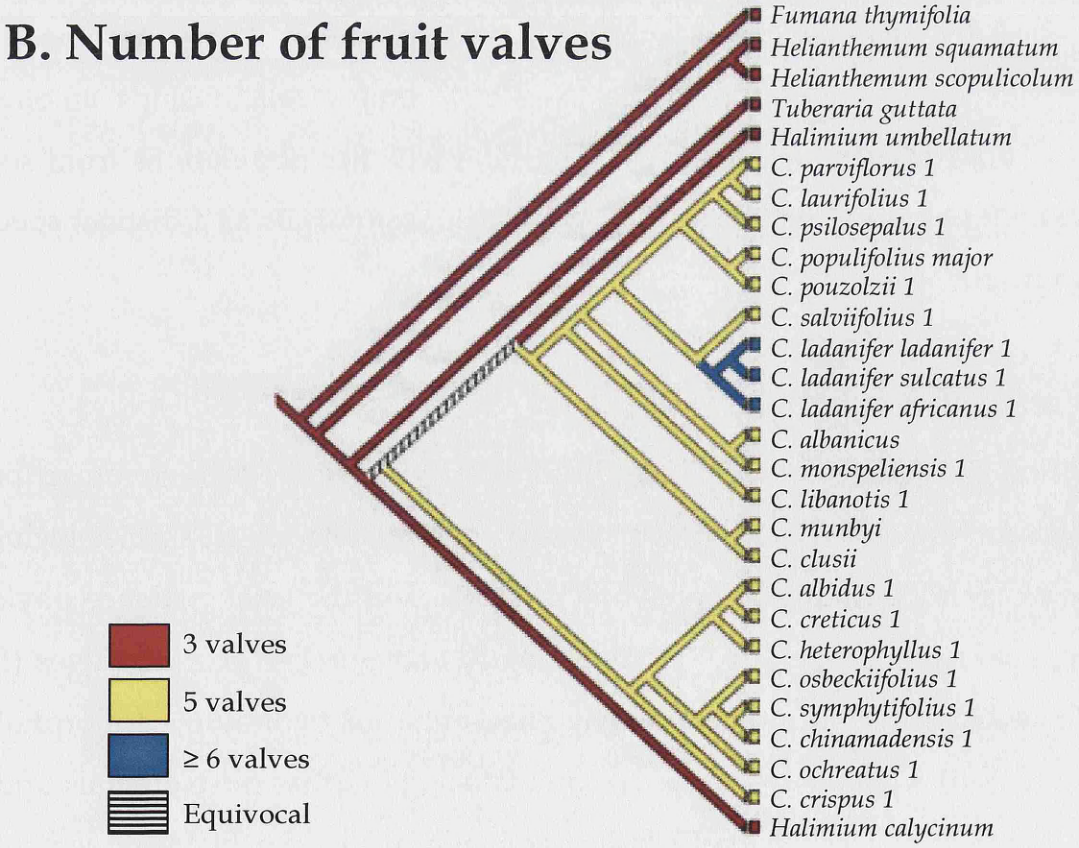
Fig. 5. Hypothesis of character evolution for style length (A) and number of fruit valves (B) using sequences from one individual per taxon. The MP tree of the combined analysis of *trnL-F, matK,* and ITS sequences chosen for character reconstruction, onto which the two characters have been mapped, is congruent with the BI tree (see text) and was obtained after implementing ACCTRAN optimization of MacClade (Maddison and Maddison, 1992).


A. Style length
Fumana thymifolia
Helianthemum squamatum
Helianthemum scopulicolum
Tuberaria guttata
Halimium umbellatum
C. parviflorus 1
C. laurifolius 1
C. psilosepalus 1
C. populifolius major
C. pouzolzii 1
C. salviifolius 1
C. ladanifer ladanifer 1
C. ladanifer sulcatus 1
C. ladanifer africanus 1
C. albanicus
C. monspeliensis 1
C. libanotis 1
C. munbyi
C. clusii
C. albidus 1
C. creticus 1
C. heterophyllus 1
C. osbeckiifolius 1
C. symphytifolius 1
C. chinamadensis 1
C. ochreatus 1
C. crispus 1
Halimium calycinum
Equivocal


B. Number of fruit valves
Fumana thymifolia
Helianthemum squamatum
Helianthemum scopulicolum
Tuberaria guttata
Halimium umbellatum
C. parviflorus 1
C. laurifolius 1
C. psilosepalus 1
C. populifolius major
C. pouzolzii 1
C. salviifolius 1
C. ladanifer ladanifer 1
C. ladanifer sulcatus 1
C. ladanifer africanus 1
C. albanicus
C. monspeliensis 1
C. libanotis 1
C. munbyi
C. clusii
C. albidus 1
C. creticus 1
C. heterophyllus 1
C. osbeckiifolius 1
C. symphytifolius 1
C. chinamadensis 1
C. ochreatus 1
C. crispus 1
Halimium calycinum
3 valves
5 valves
≥ 6 valves
Equivocal

species with purple (subgenus Cistus) and white (subgenera Leucocistus) flowers is partly supported as *Cistus parviflorus* appears in all analyses as the only purple-flowered species placed in an otherwise white-flowered lineage. Its distinctiveness has been historically recognized by creating a monotypic, supraspecific taxon (section, subgenus, genus) for this species as Ledonella. The recognition of a group of Canarian species, as a supraspecific taxon (usually called Rhodocistus) within *Cistus* subgenus Cistus, accords with the well-diagnosed natural group of long-styled species exclusive to the Canary Islands (Fig. 4). One more supraspecific taxon (section Erythrocistus), consisting of *C. albidus*, *C. creticus*, *C. heterophyllus*, and *C. crispus*, is paraphyletic because the Canarian lineage originated from a most recent common ancestor to only three of them. At a finer level of taxonomic resolution, this study supports present-day delimitation of some species (*C. parviflorus*, *C. salviifolius*, *C. libanotis*, *C. psilosepalus*, *C. ladanifer*, *C. laurifolius*, *C. pouzolzii*, *C. monspeliensis*). Our population sample, although limited, indicates that neither paraphyly nor polyphyly affect species formation in *Cistus*. One more lineage consisting of the three subspecies of *Cistus ladanifer*, as circumscribed by Demoly and Montserrat (1993), receives strong support (Fig. 4). However, our phylogenetic hypothesis does not resolve relationships among these subspecies and our data are unable to determine whether populations from southern Portugal should be recognized as *C. ladanifer* subsp. *sulcatus* or as a distinct species (*C. palinhae* Ingram).

4.2. Evolution of morphological characters

Ten morphological characters have been traditionally considered for circumscription of *Cistus*. Data for some of these characters were not available for all species included in the study (Appendix 2), but five are shown mapped on the total-evidence phylogeny: leaf base, sepal number, petal color, style length, and number of fruit valves (Fig. 4). MacClade reconstructions indicate a dynamic course of evolution of morphological characters (Fig. 5). Our phylogenetic hypothesis suggests that purple petals appear to originate twice in the *Halimium-Cistus* assemblage, result partly in agreement with taxonomic treatments since petal color serves to define a natural group of all purple-

flowered species, except for *C. parviflorus*. Leaf bases experienced multiple changes not only in *Cistus* lineages, but also in the Cistaceae and potentially within a single species (*C. ladanifer*). Three and five sepals are found across the Cistaceae reflecting multiple shifts in many groups. Five sepals are, however, maintained within the lineage of purple-flowered species. Our data suggest that the four states of style length (sessile, shorter, similar, and longer) did not evolve in a unidirectional manner in either Cistaceae or the *Cistus-Halimium* assemblage (Fig. 5A). Stigmas exceeding stamens occurs only in the Canarian species of *Cistus* and appears to have evolved from an ancestor in the lineage of purple-flowered species with styles equal in length to the stamens. The optimization of long styles is consistent with an early acquisition of this unique character state, which was then maintained during the course of speciation in the Canary Islands. It is intriguing to interpret prominent stigmas in the Canarian *Cistus* as evolution of a trait related to particular environment conditions of oceanic islands. The occurrence of long styles in other continental Cistaceae indicates recurrent acquisition of this character (Fig. 5A).

Historical reconstruction of the evolution of the number of fruit valves provides evidence of equivocal transition from three valves, consistently displayed in over 180 species of the Cistaceae, to five valves in *Cistus* (Fig. 5B). A further step resulted in the increment of fruit valves to 6-12 exclusively in a single species (*C. ladanifer*). A consistent number of fruit valves is not only exhibited within species of the Cistaceae but also within genera of closely related families (Nandi, 1998). *Cistus ladanifer* constitutes then a remarkable species model to explore multiplication of fruit valves during the development of the ovary wall. Maximum ITS sequence divergence (0.93% K-2-p distance) of extant subspecies, in comparison to another angiosperms (Richardson et al., 2001), suggests that the multi-valved fruit of *C. ladanifer* evolved following establishment of the Mediterranean climate 2.8 Ma (Suc, 1984) and after plants with 5-valved fruits had been in existence for million of years (see below). Although this character may be subject to evolutionary processes, variation between 6 and 12 valves should be studied in a broader sense because the highest number of

valves appears to be environment dependent in some populations of *C. ladanifer* subsp. *ladanifer* (Narbona, Guzmán and Vargas, unpublished data).

4.3. Hybridization and evolution in Cistus

Reproductive mechanisms that act to prevent mating occur at the individual level (self-incompatibility) in many species of *Cistus* and *Halimium* (Dansereau, 1940; Herrera, 1992). Consequently, outcrossing favors both inter-individual and inter-specific production of hybrids. Artificial hybridization experiments carried out between 1860 and 1868 by Bornet (Gard, 1910, 1913, 1914) illustrate the facility in which species of *Cistus* generate F_1, F_2, F_3, and F_4 offspring. Gard successfully undertook crosses between six species of *Cistus* that gave rise to fertile progeny (Gard, 1910). Formation of an intergeneric hybrid between *Cistus* and *Halimium* (x *Halimiocistus*) demonstrates even a wider range of genetic compatibility. Natural hybridization is common as species pairs of *Cistus* occur in sympatry. For instance, 44 natural hybrids between varieties and species were recorded by Dansereau (1940) and 20 inter-specific hybrids of *Cistus* have been described from the Iberian Peninsula (Martín and Guinea, 1949; Demoly and Montserrat, 1993). In light of these results, hybridization was hypothesized as the major mode of evolution in *Cistus* since the early 20th century (Dansereau, 1940; Demoly, 1996). Molecular evidence for hybridization is manifested by (1) nucleotide double-peak patterns (additivity) in 10 ITS sequences, (2) more than two ITS copies of different length size from PCR amplifications of the same DNA samples, and (3) five of the 15 nucleotide additivity sites are found at parsimony-informative positions. This, together with the biparental inheritance of the ITS region, is interpreted as an argument of alternative ITS copies inherited by two or more parental donors (Fuertes et al., 1999), followed by failure to fully homogenize (concerted evolution) multiple ITS copies in the nuclear genome over generations (Rauscher et al., 2002). Apart from success in obtaining artificial inter-species crossings and detecting a molecular pattern of sequence additivity in *Cistus*, the occurrence of hybrid swarms in certain locations of Morocco and Spain (unpublished data) support the viability of hybridization as an evolutionary mechanism in *Cistus*.

Conflicting signals observed from phylogenies of recombinant, biparental nuclear ribosomal ITS vs. non-recombinant, uniparental organelles have typically been interpreted as evidence for extensive reticulation processes at the species level (Wendel and Doyle, 1998). Unfortunately, the limited resolution obtained in the ITS phylogeny preclude detecting fundamental discordance with the plastid phylogeny (Fig. 3). A potential case of speciation by hybridization should be further explored in *C. parviflorus*, as suggested by the detection of sequence additivity (ITS additivity of nucleotides in two sites and different-length sequences in one accession) and the combination of two morphological characters within this species that otherwise define exclusively the two *Cistus* lineages, i.e. purple petals and sessile stigmas. While incongruence between nuclear and organelle genomes may reveal evidence for reticulation, the converse is not necessarily true based only on ITS sequences (Chase et al., 2003). The use of alternative nuclear markers, such as single-copy genes (already in progress), may shed further light on whether balanced concerted evolution of ITS sequences impede obtaining full resolved phylogenies (Nieto Feliner et al., 2001).

4.4. Differentiation in the Mediterranean

Cistus exhibits a dominant role in woodland understory and evergreen scrub of the Mediterranean region (Médail and Quézel, 1997). The major center of species diversity is in the western Mediterranean, particularly on both sides of the Strait of Gibraltar (14 of 20 in Andalusia and northern Morocco (Fig. 1)). The same is true for *Halimium* (the closest genus to *Cistus*), with most species (8 of 10) distributed in this area. Early differentiation of present-day *Cistus* may have occurred in the western Mediterranean based on the following molecular evidence: (1) the total-evidence phylogeny reveals that the six species of *Cistus* exclusively occurring in the eastern Mediterranean and the Canary Islands (*C. albanicus* in Albania and Greece; *C. parviflorus* in Greece, Turkey, Italy, Cyprus, and Libya; *C. chinamadensis, C. ochreatus, C. osbeckiifolius, C. symphytifolius* in the Canary Islands) do not form basal-most sister groups (Fig. 4); (2) a western-Mediterranean species (*C. crispus*) is sister to the remaining species of the purple-flowered lineage (excluding *C. parviflorus*), as well as two western-Mediterranean

species (*C. clusii, C. munbyi*) to the white-flowered lineage; (3) the 14 species distributed in the western Mediterranean reach levels of pair-wise sequence divergence similar to those within the whole genus (1.62 vs. 1.78% in *trnK-mat*K; 2.88 vs. 3.15% in *trnL-F;* 4.36 vs. 4.86% in ITS (Table 2)). High morphological (taxonomy) and molecular (phylogenetics) divergence in the western Mediterranean and Macaronesia suggests a prime hotspot of diversity not only in *Cistus* but also in disparate angiosperms (Médail and Quézel, 1997). It has been suggested that regional diversity in mediterranean-climatic regions is the product of local diversity and differentiation diversity in relation to environmental heterogeneity (Cowling et al., 1996; Thompson, 2005). *Cistus* species do not fall into this diversity pattern in spite of absence of a long-distance dispersal syndrome (dry capsules and seeds) and environmental specificity referring to acidic and carbonate substrates. In fact, there are no endemics to particular Mediterranean countries and no pattern of geographic cohesion (Fig. 4). Dispersal and colonization of *Cistus* across areas in the Mediterranean basin is inferred to have taken placed successfully after divergence and species formation. Besides wide distribution of most species, the occurrence of circum-Mediterranean species (*C. creticus, C. monspeliensis, C. salviifolius*) in the two major lineages supports this view.

The oldest pollen record for Cistaceae (*Cistacearumpollenites*) dates from the Lower Miocene from Czechia (Bohemia) (Konzalova, 1967). This identification should be taken cautiously as there are difficulties in identifying pollen samples at the genus level; identification of species is, however, most reliable once determining adscription to *Cistus* (Ukraintseva, 1991). In contrast, *Cistus* displays an unequivocal shape and number of fruit valves (Appendix 1) in the Mediterranean flora. Fruits in the amber-bearing sands of the Baltic Sea (Zemland) and Germany (Montbauer) from the Oligocene (Palibin, 1909) provide reliable evidence for a distribution of *Cistus* not restricted to the present-day Mediterranean region. Objections about inference of centers of origin have been extensively discussed (Bremer, 1992). Despite present-day distribution and diversity of *Cistus* species, paleobotanical data strongly suggest centers of origin for *Cistus* out of the Mediterranean region, (as nowadays outlined, Fig. 1), and

a time of formation of at least 23 million years ago. We cannot consider calibrated divergence times for *Cistus* because no absolute substitution rate (molecular clock) has been estimated in the Cistaceae. Using both mean values of ITS divergence (4.37×10^{-9} nucleotide substitution/site/year) in angiosperms (Richardson et al., 2001) and the maximum ITS sequence divergence found within *Cistus* (4.7% K-2-p divergence), we infer that differentiation of extant species of *Cistus* might not predate 8-7 Ma. If this result is consistently obtained in future investigations, as increasing the sample and using alternative markers and molecular-clock methods, it would be plausible a hypothesis of that present-day species differentiation occurred much later than the formation of *Cistus*.

4.5. Historical biogeography of Canarian species

Floristic affinities, number of introductions, time of dispersal, and speciation patterns have been the major objectives inferred for Macaronesian plant groups by means of molecular phylogenetics (Carine et al., 2004). The biogeographic results presented in this paper support general patterns of plant colonization in the Canary Islands. The species of *Cistus* endemic to the Canary Islands are imbedded in the purple-flowered lineage in the total-evidence analysis (Fig. 4). Both plastid and nuclear phylogenies reveal a single colonization of *Cistus* in the Canary Islands to account for present-day differentiation into four species (*C. ochreatus, C. chinamadensis, C. osbeckiifolius, C. symphytifolius*) (Fig. 3). Despite a significant number of phylogenies including Macaronesian taxa, few of them use molecular data from different cellular genomes and, where they have been used, few are congruent with the placement of Canarian lineages (Francisco-Ortega, 2004). We herein provide strong multigenome evidence for a single introduction of purple-flowered *Cistus* in the Canary Islands. Single introductions in Macaronesia appear to be the rule rather than the exception for plant groups consisting of numerous species (Carine et al., 2004; Silvertown, 2004; Vargas, 2005), although topological congruence between organullar and nuclear markers and a larger number of examples are needed. One question that remains to be resolved is

whether the occurrence of the white-flowered species *C. monspeliensis* in the Canary Islands and Madeira is the result of natural or human-influenced introduction.

Phylogenetic reconstructions place the Canarian endemics of *Cistus* in a clade with three purple-flowered continental species (*C. albidus, C. heterophyllus, C. creticus*). A set of morphological attributes, such us petiolate leaves and a high number of stamens, appear to relate *C. heterophyllus* to the Canarian species (Table 3). This species is currently distributed in the western Mediterranean supporting the close floristic relationship between the Canary Islands and the Mediterranean (Carine et al., 2004), and particularly with north-western Africa since *C. heterophyllus* occurs almost exclusively in Morocco and Algeria. Irrespective of the closest, extant relative of the Canarian lineage, character-state reconstruction using MacClade reveals that the four species endemic to the Canary Islands (*C. ochreatus, C. chinamadensis, C. osbeckiifolius, C. symphytifolius*) originated from a 5-sepaled, purple-flowered, mid-styled, and 5 fruit-valved ancestor (Fig. 4, 5). Once *Cistus* colonized and established in the archipelago, speciation took place in conjunction with maintenance of long styles exceeding stamens (Fig. 5A).

Previous allozyme diversity results (Batista et al., 2001) are in agreement with the levels of nucleotide divergence found in the present study, in which *C. symphytifolius* displays the highest levels of K-2-p pair-wise divergence with respect to the other Canarian species: 0.32% for ITS (between *C. symphytifolius* 2 and *C. chinamadensis* 1); 0.00% for *trnK-matK;* and 0.71% for *trnL-F* (between *C. chinamadensis* and *C. osbeckiifolius*). Additionally, populations of *C. symphytifolius* from different islands display a polyphyletic pattern and the highest levels of nucleotide pair-wise divergence for these three markers (unpublished data). Given that Tenerife and Gran Canaria harbor the four species of *Cistus* and the highest levels of molecular diversity, including isozyme and nucleotide divergence, we hypothesize that *Cistus* lineages from these two islands have spawned new lines of evolution via interisland dispersal. Two major evolutionary models have been described to explain speciation of angiosperms in the Canary archipelago: interisland dispersal followed by speciation and intraisland

radiation followed by dispersal to similar habitats (Baldwin et al., 1998). The bulk of molecular evidence and present-day distributions suggest extensive interisland dispersal of *C. symphytifolius*, or a closely-related ancestor, followed by differentiation of new taxa in some islands (Batista et al., 2001). These conclusions require further intraspecific sampling of *C. symphytifolius* from every island.

Accelerated morphological diversification has been hypothesized for insular plant groups commonly regarded as examples of explosive radiation in which sequence identity is maintained (Baldwin et al., 1998). We do not hypothesize rapid speciation for *Cistus* because of a low number of Canarian species (five including a recently described species by Demoly (2004)) separated by considerable tree branch length with respect to continental species. Levels of molecular divergence of Canarian and their closest continental relatives (0.94 % for ITS; 0.47% for *trnK-matK*; 0.71% for *trnL-F*) suggest a relatively old colonization, which contrasts with similar habit (shrubs) as that of their closest relatives in the continent. Differentiation of genera colonizing oceanic islands resulted in woody plants in a numerous number of plant groups, including remarkable shifts from herbs to a woody condition (Baldwin et al., 1998). Given a considerable time for establishment of *Cistus* in the Canary Islands, it is intriguing to observe neither an increment in size (woodiness) nor occupation of new habitats. However, competition-free environments were likely to be rare by the time of *Cistus* establishment, as inferred by low sequence divergence with regard to the origin of the oldest island (Fuerteventura, 20.7 Ma) (Silvertown, 2004). In addition, limited capability of *Cistus* to occupy different habitats in the continent may be related to failure in exploitation of new, diverse habitats in oceanic islands. The four species of *Cistus* inhabit Canarian woodlands as understory, and form part of successional stages of Mediterranean and pine tree communities, therefore similar in ecology to their continental congeners (Ceballos and Ortuño, 1976). Lineages of two broom genera (*Adenocarpus, Teline*) also exhibit adaptation to woodland understory with no shift in woodiness, limited exploitation of new ecological niches, and similar levels of ITS sequence divergence related to species number (Percy and Cronk, 2002).

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References

Alfaro, M.E., Zoller, S., Lutzoni, F., 2003. Bayes or bootstrap? A simulation study comparing the performance of bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Mol. Biol. Evol. 20(2), 255–266.

Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phyl. Evol. 29, 417–434.

Arrington, J.M., Kubitzki, K., 2003. Cistaceae. In: Kubitzki, K. (Ed.), The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales. Springer, Berlin, pp. 62–70.

Baldwin, B.G., Crawford, D.J., Francisco-Ortega, J., Kim, S-C., Sang, T., Stuessy, T.F., 1998. Molecular phylogenetic insights on the origin and evolution of oceanic island plants. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer academic Publishers, Boston, pp. 410–441.

Batista, F., Bañares, A., Caujapé-Castells, J., Carqué, E., Marrero-Gómez, M., Sosa, P.A., 2001. Allozyme diversity in three endemic species of *Cistus* (Cistaceae) from the Canary Islands: intraspecific and interspecific comparisons and implications for genetic conservation. Am. J. Bot. 88(9), 1582–1592.

Bosch, J., 1992. Floral biology and pollinators of three co-occurring *Cistus* species (Cistaceae). Bot. J. Linn. Soc. 109, 39–55.

Bremer, K., 1992. Ancestral areas: A cladistic reinterpretation of the center of origin concept. Syst. Biol. 41(4), 436–445.

Carine, M.A., Russell, S.J., Santos-Guerra, A., Francisco-Ortega, J., 2004. Relationships of the Macaronesian and Mediterranean floras: molecular evidence for multiple colonizations into

Macaronesia and back-colonization of the continent in *Convolvulus* (Convolvulaceae). Am. J. Bot. 91, 1070–1085.

Ceballos, L., Ortuño, F., 1976. Estudio sobre la vegetación y flora forestal de Las Canarias occidentales. Excelentísimo Cabildo Insular, Santa Cruz de Tenerife, 433 pp.

Chase, M.W., Knapp, S., Cox, A.V., Clarkson, J.J., Butsko, Y., Joseph, J., Savolainen, V., Parokonny, A. S., 2003. Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). Ann. Bot. 92: 107–127.

Cowling, R.M., Rundel, P.W., Lamont, B.B. Arroyo, M.K., Arianoutsou, M. 1996. Plant diversity in Mediterranean-climate regions. TREE 11(9): 362–366.

Dansereau, P.M., 1939. Monographie du genre *Cistus*. Boissiera 4, 1–90.

Dansereau, P., 1940. Études sur les hybrides de Cistes. Ann. Épiphyt., ser. 2, 6, 7–26.

Demoly, J.P., Montserrat, P., 1993. *Cistus*. In: Castroviejo, S. *et al.* (Eds.), Flora Ibérica, vol. 3. Consejo de Investigaciones Cientifícas, Madrid, pp. 319–337.

Demoly, J. P., 1996. Les hybrides binaires rares du genre *Cistus* L. (*Cistaceae*). Anales Jard. Bot. Madrid 54, 241–254.

Demoly, J.P., 2004. Une nouvelle espèce du genre *Cistus* L. (Cistaceae) endémique de l'île de Grande Canarie (Espagne). Acta Bot. Gallica 151(2), 231–232.

Ducousso, M., Béna, G., Bourgeois, C., Buyck, B., Eyssartier, G., Vincelette, M., Rabevohitra, L., Randrihasipara, L., Dreyfus, B., Prin, Y., 2004. The last common ancestor of Sarcolaenaceae and Asian dipterocarp trees was ectomycorrhizal before the India-Madagascar separation, about 88 million years ago. Mol. Ecol. 13, 231–236.

Dunal, 1824. Cistineae in De Candolle. A. P. (Ed.), Prodromus Systematis Naturalis Regni Vegetabilis I, Treuttel et Würtz, Paris, pp. 263–286.

Ellul, P., Boscaiu, M., Vicente, O., Moreno, V., Rosselló, J.A., 2002. Intra- and interspecific variation in DNA content in *Cistus* (Cistaceae). Ann. Bot. 90, 345–351.

Francisco-Ortega, J., 2004. Origin of the Macaronesian flora and its relationships with the Mediterranean flora. In: IX meeting International Organization of Plant Biosystematics. Plant Evolution in Mediterranean climate zones, May, 16-19, Valencia, Spain.

Fuertes, J., Rosselló, J.A., Nieto, G., 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). Mol. Ecol. 8, 1341–1346.

Gard, M., 1910. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Premier mémoire: notes inédites et résultats expérimentaux. Ann. Sci. Nat. ser. 9, 12, pp. 71–115.

Gard, M., 1913. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Deuxième mémoire: les espèces et les hybrides binaires. Beih. Bot. Centralbl. 29, pp. 306–394.

Gard, M., 1914. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Troisième mémoire: Les hybrides dérivés et les hybrides complexes. Beih. Bot. Centralbl. 31, pp. 373–428.

Grosser, W., 1903. Cistaceae. In: Engler, A. (Ed.), Dans Pflanzenreich 14, Berlin, pp. 161.

Herrera, J., 1986. Flowering and fruiting phenology in the coastal shrublands of Doñana, south Spain. Vegetatio 68, 91–98.

Herrera, J., 1987. Flower and fruit biology in southern Spanish Mediterranean shrublands. Ann. Missouri Bot. Gard. 74(1), 69–78.

Herrera, J., 1992. Flower variation and breeding systems in the *Cistaceae*. Plant Syst. Evol. 179, 245–255.

Johnson, L.A., Soltis, D.E., 1994. matK DNA and phylogenetic reconstruction in Saxifragaceae s. str. Syst. Bot. 19(1), 143–156.

Johnson, L.A., Soltis, D.E., 1998. Assessing congruence: empirical examples from molecular data. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer academic Publishers, Boston, pp. 297–343.

Kelchner, S.A., 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. Ann. Missouri Bot. Gard. 87, 482–498.

Konzalova, M., 1967. Paleontologicke zprava k lokalite. Archiv Geoindustria, Geofond, Prague.

Maddison, W.P., Maddison, D.R., 1992. MacClade: Analysis of phylogeny and character evolution. Version 3.01. Sinauer Associates, Sunderland, Massachusetts.

Martín, M., Guinea, E., 1949. Jarales y jaras (Cistografía Hispánica). Bol. Inst. Forest. Invest. Exp. 49, Madrid, 228 pp.

Médail, F., Quézel, P. 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. Ann. Missouri Bot. Gard. 84: 112–127.

Nandi, O.I., 1998. Ovule and seed anatomy of *Cistaceae* and related *Malvanae*. Pl. Syst. Evol. 209, 239–264.

Nieto Feliner, G., Fuertes, J., Rosselló, J.A., 2001. Can extensive reticulation and concerted evolution result in a cladistically structured molecular data set? Cladistics 17, 301–312.

Nylander, J.A.A., 2002. MrModeltest v1.0b. Department of Systematic Zoology, Uppsala University, (program distributed by the author).

Palibin, I.V., 1909. Cistaceae. In: Kuznetsov, N., Bush, N., Fomin, A. (Eds.). Flora Caucasica Critica, vol. 3, pp. 117–123.

Percy, D.M., Cronk, Q.C.B. 2002. Different fates of island brooms: contrasting evolution in *Adenocarpus, Genista,* and *Teline* (Genisteae, Fabaceae) in the Canary Islands and Madeira. Am. J. Bot. 89(5): 854–864.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14(9), 817–818.

Rauscher J.T., Doyle J.J., Brown H.D., 2002. Internal transcribed spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploid complex. Mol. Ecol. 11, 2691–2702.

Richardson, J.E., Pennington, R.T., Pennington, T.D., Hollingsworth, P.M., 2001. Rapid diversification of a species-rich genus of neotropical rain forest trees. Science 293, 2242–2245.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Roy, J., Sonié, L., 1992. Germination and population dynamics of *Cistus* species in relation to fire. J. Appl. Ecol. 29, 647–655.

Saenz, C. 1979. Pollen morphology of Spanish Cistaceae. Grana 18: 91–98.

Salamin, N. Chase, M.W., Hodkinson, T.R., Savolainen, V. 2003. Assessing internal support with large phylogenetic DNA matrices. Mol. Phyl. Evol. 27: 528–539.

Savolainen, V., Fay, M.F., Albach, D.C., Backlund, A., van der Bank, M., Cameron, K.M., Johnson, S.A., Lledó, M.D., Pintaud, J.C., Powell, M., Sheahan, M.C., Soltis, D.E., Soltis, P.S., Weston, P., Whitten, W.M., Wurdack, K.J., Chase, M.W., 2000. Phylogeny of the eudicots: a nearly complete familial analysis based on *rbcL* gene sequences. Kew Bulletin 55, 257–309.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49(2), 369–381.

Silvertown J. 2004. The ghost of competition past in the phylogeny of island endemic plants. J. Ecol. 62: 168–173.

Soltis, D.E., Soltis, P.S., Chase, M.W., Mort, M.E., Albach, D.C., Zanis, M., Savolainen, V., Hahn, W.H., Hoot, S.B., Fay, M.F., Axtell, M., Swensen, S.M., Prince, L.M., Kress, W.J., Nixon, K.C., Farris, J.S., 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL,* and *atpB* sequences. Bot. J. Linn. Soc. 133, 381–461.

Soriano, I. 2002. Cistaceae. In: Valdés, B., Rejdali, M., Achhal, A., Jury, J.L., Montserrat, J.M. (Eds.). Checklist of vascular plants of N Morocco with identification keys, vol. 2. Consejo Superior de Investigaciones Científicas, Madrid, pp. 196–200.

Spach, E. 1836. Conspectus monographiae Cistacearum. Ann. Sci. Nat. Bot. 6: 357-375.

Suc, J.P., 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307, 429–432.

Sun, Y., Skinner, D.Z., Liang, G.H., Hulbert, S.H., 1994. Phylogenetic analysis of *Sorghum* and related taxa using Internal Transcribed Spacer of nuclear ribosomal DNA. Theor. Appl. Genet. 89, 26–32.

Swofford, D. L. 1999, PAUP*: Phylogenetic Analysis Using Parsimony. Version 4.07b: Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.

Taberlet, I., Gielly, L., Pautou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17, 1105–1109.

Talavera, S., Gibbs, P.E., Herrera, J., 1993. Reproductive biology of *Cistus ladanifer* (*Cistaceae*). Pl. Syst. Evol. 186, 123–134.

Templeton, A.R., 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37(2), 221–244.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25(24), 4876–4882.

Thompson, J.D. 2005. Plant evolution in the Mediterranean. Oxford University Press, Oxford. 288 pp.

Trabaud, L., Renard, P., 1999. Do light and litter influence the recruitment of *Cistus* spp. stands? Israel J. Plant Sci. 47, 1–9.

Ukraintseva, V.V., 1991. The palynological study of the genus *Cistus*. Botanicheskii Zhurnal 76, 979–985.

Vargas, P., 2007. Are Macaronesian islands refugia of relict plant lineages?: a molecular survey. In: Weiss, S.J., Ferrand, N. (Eds.), Phylogeography in Southern European Refugia: evolutionary perspectives on the origins and conservation of European biodiversity. Springer, Dordrecht, pp. 297-314.

Warburg, E.F., 1968. *Cistus* L. In: Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A. (Eds.), Flora Europaea, vol. 2. Cambridge University Press, Cambridge, pp. 282–285.

Wendel, J.F., Doyle, J.J., 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer academic Publishers, Boston, pp. 265–296.

White, T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Geldfand, D., Sninsky, J., White, T. (Eds.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp. 315–322.

Willkomm, M., 1856. Cistinearum orbis veteris descriptio monographica. Icones et Descriptiones Plantarum vol. II.

Appendix 1

Table of geographical distribution, material source, voucher, and GenBank accession numbers

Appendix 1. Cistaceae taxa sequenced for ITS, *trnL-F*, and *trnK-matK*. Geographic distribution, material source, voucher, and GenBank accession numb are also included. Taxonomy follows Demoly and Montserrat (1993) and local floras

Taxon	*Distribution*	*Locality/source*	*Voucher*
Cistus L.			
Cistus albanicus E.F. Warb. ex Heywood	Albania, Greece	Cultivated	R. G. Page 8cBGA04 (M
Cistus albidus L.	Iberia, S France, N Italy, N Africa, Corsica, Sardigna	Spain, Madrid, Aldea del Fresno (1)	P. Vargas 25PV03 (MA)
"	"	Morocco, Tetuán (2)	P. Vargas 41PV03 (MA)
Cistus chinamadensis Bañares et Romero	La Gomera, Tenerife (Canary Islands)	Canary Islands, San Sebastián de La Gomera (1)	Á. Fernández & J. Leralt 44BGA04 (MA)
"	"	Canary Islands, La Gomera (2)	R. G. Page 144BGA04 (M
Cistus clusii Dunal	Spain, Italia, N Africa, Sicily	Spain, Málaga, Mijas	R. G. Page 8bBGA04 (M
Cistus creticus L.	Mediterranean Basin	Greece, Kineta (1)	P. Vargas 392PV02 (MA
"	"	Greece, Olympus (2)	P. Vargas 209PV04 (MA
Cistus crispus L.	Iberia, S France, N Italy, N Africa, Corsica, Sicily	Spain, Córdoba, Posadas (1)	B. Guzmán 58BGA04 (M
"	"	Morocco, Grottes d'Hercules (2)	P. Vargas 32PV03 (MA)
Cistus heterophyllus Desf.	SE Spain, N Africa	Morocco (1)	O. Filippi 7BGA04 (MA
"	"	Morocco, Beni-Hadifa (2)	B. Guzmán 99BGA04 (M
Cistus ladanifer L. ssp. *africanus* Dans.	S Spain, N Africa	Morocco, Grottes d'Hercules (1)	P. Vargas 28PV03 (MA)
"	"	Morocco, Targuist (2)	B. Guzmán 109BGA04 (
Cistus ladanifer L. ssp. *ladanifer*	S France, Iberia, N Africa, Cyprus	Spain, Madrid, Boadilla del Monte (1)	B. Guzmán 7BGA03 (M
"	"	Spain, Almería, Sierra de la Alhamilla (2)	P. Vargas 179PV04 (MA
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts.	S Portugal	Portugal, Cabo San Vicente (1)	B. Guzmán 36BGA04 (M
"	"	Portugal, Sagres (2)	B. Guzmán 29BGA04 (M
Cistus laurifolius L.	N Africa, Iberia, France, Italy, Corsica, Turkey	Spain, Madrid, Las Rozas (1)	P. Vargas 12PV03 (MA)
"	"	Spain, Jaén, Sierra de Segura (2)	B. Guzmán 13BGA03 (M
Cistus libanotis L.	Portugal, S Spain, Argelia	Cultivated (1)	O. Filippi 5BGA04 (MA
"	"	Spain, Córdoba (2)	R. G. Page 149BGA04 (
Cistus monspeliensis L.	Mediterranean Basin, Canary Islands	Morocco, Grottes d'Hercules (1)	P. Vargas 30PV03 (MA)
"	"	Portual, Sagres (2)	B. Guzmán 35BGA04 (M
Cistus munbyi Pomel	Algeria, Morocco	Morocco	O. Filippi 4BGA04 (MA
Cistus ochreatus C. Sm. ex Buch	Gran Canaria (Canary Islands)	Canary Islands, Gran Canaria (1)	R. G. Page 150BGA04 (M
"	"	Canary Islands, Gran Canaria (2)	R. G. Page 8BGA04 (MA
Cistus osbeckiifolius Webb ex Christ	Tenerife (Canary Islands)	Canary Islands, Tenerife (1)	O. Filippi 160BGA04 (M
"	"	Canary Islands, Tenerife, La Fortaleza (2)	S. Castroviejo (471793 M
Cistus parviflorus Lam.	Greece, Turkey, Italy, Cyprus, N Libia, Lampedusa	Greece, Crete (1)	O. Filippi 6BGA04 (MA
"	"	Cyprus (2)	R. G. Page 151BGA04 (M
Cistus populifolius L. ssp. *populifolius*	Iberia, S France	Spain, Ávila, Arenas de San Pedro	P. Vargas 5PV03 (MA)
Cistus populifolius L. ssp. *major* (Dunal) Heywood	Iberia, N Morocco	Portugal, Ourique	B. Guzmán 20BGA04 (M

Appendix 1. *(Continued)*

Cistus pouzolzii Delile	Algeria, N Morocco, France	France (1)	R. G. Page 8tBGA04 (M
"	"	Morocco, Ketama (2)	S. L. Jury 698247MA
Cistus psilosepalus Sweet	Iberia, France	Spain, Ávila, Arenas de San Pedro (1)	P. Vargas 7PV03 (MA)
"	"	Spain, Lugo (2)	R. G. Page 147BGA04 (M
Cistus salviifolius L.	Mediterranean Basin	Spain, Ávila, Arenas de San Pedro (1)	P. Vargas 6PV03 (MA)
"	"	Spain, Granada, Sierra Nevada (2)	P. Vargas 119PV04 (MA
Cistus symphytifolius Lam.	El Hierro,La Palma,La Gomera,Tenerife,Gran Canaria	Canary Islands, La Palma, Punta Gorda (1)	P. Vargas 263PV02 (MA
"	"	Canary Islands, La Palma, La Cumbrecita (2)	B. Guzmán 143BGA04 (M
Fumana (Dunal) Spach			
Fumana thymifolia (L.) Spach ex Webb	Mediterranean Basin	Portugal, Ferrerías	B. Guzmán 53BGA04 (M
Halimium (Dunal) Spach			
Halimium calycinum (L.) K. Koch	Iberia, NW Morocco	Portugal, Cabo Sardao	B. Guzmán 49BGA04 (M
Halimium umbellatum (L.) Spach	Mediterranean Basin	Spain, Madrid, Tres Cantos	P. Vargas 71BGA04 (MA
Helianthemum Mill.			
Helianthemum scopulicolum L.	Balearic Islands	Cultivated	B. Guzmán 67BGA04 (M
Helianthemum squamatum (L.) Dum. Cours.	Iberia, N Africa	Cultivated	B. Guzmán 70BGA04 (M
Tuberaria Dunal			
Tuberaria guttata (L.) Fourr.	W Europe, Mediterranean Basin, Canary Islands	Portugal, Vila do Bispo	B. Guzmán 44BGA04 (M

Appendix 1. *(Continued)*

Taxon	*ITS accession no.*	trnL-F *accession no.*	*trnK*-matK *accession no.*
Cistus			
Cistus albanicus E.F. Warb. ex Heywood	DQ092964	DQ093057	DQ093010
Cistus albidus L. (1)	DQ092932	DQ093021	DQ092974
Cistus albidus L. (2)	DQ092933	DQ093022	DQ092975
Cistus chinamadensis Bañares et Romero (1)	DQ092942	DQ093033	DQ092986
Cistus chinamadensis Bañares et Romero (2)	DQ092943	DQ093034	DQ092987
Cistus clusii Dunal	DQ092963	DQ093056	DQ093009
Cistus creticus L. (1)	DQ092937	DQ093026	DQ092979
Cistus creticus L. (2)	DQ092936	DQ093025	DQ092978
Cistus crispus L. (1)	DQ092967	DQ093060	DQ093013
Cistus crispus L. (2)	-	DQ093061	DQ093014
Cistus heterophyllus Desf. (1)	DQ092944	DQ093035	DQ092988
Cistus heterophyllus Desf. (2)	-	DQ093036	DQ092989
Cistus ladanifer L. ssp. *africanus* Dans. (1)	DQ092955	DQ093047	DQ093000
Cistus ladanifer L. ssp. *africanus* Dans. (2)	DQ092956	DQ093048	DQ093001
Cistus ladanifer L. ssp. *ladanifer* (1)	DQ092951	DQ093043	DQ092996
Cistus ladanifer L. ssp. *ladanifer* (2)	DQ092952	DQ093044	DQ092997
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts. (1)	DQ092953	DQ093045	DQ092998
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts. (2)	DQ092954	DQ093046	DQ092999
Cistus laurifolius L. (1)	DQ092958	DQ093051	DQ093004
Cistus laurifolius L. (2)	DQ092959	DQ093052	DQ093005
Cistus libanotis L. (1)	DQ092947	DQ093039	DQ092992
Cistus libanotis L. (2)	DQ092948	DQ093040	DQ092993
Cistus monspeliensis L. (1)	DQ092965	DQ093058	DQ093011
Cistus monspeliensis L. (2)	DQ092966	DQ093059	DQ093012
Cistus munbyi Pomel	DQ092960	DQ093053	DQ093006
Cistus ochreatus C. Sm. ex Buch (1)	DQ092941	DQ093031	DQ092984
Cistus ochreatus C. Sm. ex Buch (2)	-	DQ093032	DQ092985
Cistus osbeckiifolius Webb ex Christ (1)	DQ092938	DQ093027	DQ092980
Cistus osbeckiifolius Webb ex Christ (2)	-	DQ093028	DQ092981
Cistus parviflorus Lam. (1)	DQ092934	DQ093023	DQ092976
Cistus parviflorus Lam. (2)	DQ092935	DQ093024	DQ092977
Cistus populifolius L. ssp. *populifolius*	-	DQ093050	DQ093003
Cistus populifolius L. ssp. *major* (Dunal) Heywood	DQ092957	DQ093049	DQ093002
Cistus pouzolzii Delile (1)	DQ092961	DQ093054	DQ093007
Cistus pouzolzii Delile (2)	DQ092962	DQ093055	DQ093008
Cistus psilosepalus Sweet (1)	DQ092949	DQ093041	DQ092994
Cistus psilosepalus Sweet (2)	DQ092950	DQ093042	DQ092995
Cistus salviifolius L. (1)	DQ092945	DQ093037	DQ092990

Appendix 1. *(Continued)*

Cistus salviifolius L. (2)	DQ092946	DQ093038	DQ092991
Cistus symphytifolius Lam. (1)	DQ092939	DQ093029	DQ092982
Cistus symphytifolius Lam. (2)	DQ092940	DQ093030	DQ092983
Fumana (Dunal) Spach			
Fumana thymifolia (L.) Spach ex Webb	DQ092926	DQ093015	DQ092968
Halimium (Dunal) Spach			
Halimium calycinum (L.) K. Koch	DQ092931	DQ093020	DQ092973
Halimium umbellatum (L.) Spach	DQ092930	DQ093019	DQ092972
Helianthemum Mill.			
Helianthemum scopulicolum L.	DQ092928	DQ093017	DQ092970
Helianthemum squamatum (L.) Dum. Cours.	DQ092927	DQ093016	DQ092969
Tuberaria Dunal			
Tuberaria guttata (L.) Fourr.	DQ092929	DQ093018	DQ092971

Appendix 2

Table of morphological characters and states

Appendix 2. Morphological characters and states on which the taxonomy of *Cistus* has been mostly based. Data were taken from basic floras, Dansereau (1939), Demoly and Montserrat (1993), and personal observations. Exine thickness and sculpturing as in Saenz (1979)

	Sepals	**Petal color**	**Leaf base** (on vegetative branches)	**Leaf shape**	**Placenta**
Subgenus *Cistus*					
Cistus albidus L.	5	Purple	Sessile	Oblong to elliptical	Polysperm
Cistus chinamadensis Bañares et Romero	5	Purple	Petiolate	Lanceolate to elliptical	-
Cistus creticus L.	5	Purple	Petiolate	Oblong to elliptical	Polysperm
Cistus crispus L.	5	Purple	Sessile	Oblong to elliptical	Polysperm
Cistus heterophyllus Desf.	5	Purple	Petiolate	Rhombic to lanceolate-elliptical	Polysperm
Cistus ochreatus C. Sm. ex Buch	5	Purple	Petiolate	Ovate-oblong	-
Cistus osbeckiifolius Webb ex Christ	5	Purple	Petiolate	Lanceolate to elliptical	-
Cistus parviflorus Lam.	5	Pink	Petiolate	Oblong to oblong-cordate	-
Cistus symphytifolius Lam.	5	Purple	Petiolate	Widely lanceolate to ovate	-
Subgenus *Leucocistus* Willk.					
Cistus albanicus E.F. Warb. ex Heywood	5	White	Subsessile	Elliptical to lanceolate-spathulate	-
Cistus ladanifer L. ssp. *africanus* Dans.	3	White	Petiolate	Lanceolate-elliptical to linear	Polysperm
Cistus ladanifer L. ssp. *ladanifer*	3	White	Sessile	Linear-lanceolate to lanceolate	Polysperm
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts.	3	White	Sessile	Elliptical to oblong	Polysperm
Cistus laurifolius L.	3	White	Petiolate	Ovate-lanceolate to ovate-cordate	Polysperm
Cistus monspeliensis L.	5	White	Sessile	Linear-lanceolate to linear-elliptical	Polysperm
Cistus populifolius L. ssp. *populifolius*	5	White	Petiolate	Cordate	Polysperm
Cistus populifolius L. ssp. *major* (Dunal) Heywood	5	White	Petiolate	Cordate	Polysperm
Cistus pouzolzii Delile	5	White	Sessile	Lanceolate to ovate-lanceolate	-
Cistus psilosepalus Sweet	5	White	Sessile	Oval-lanceolate to oblong	Polysperm
Cistus salviifolius L.	5	White	Petiolate	Ovate to ovate-oblong	Polysperm
Subgenus *Halimioides* (Willk.) Demoly & P. Monts.					
Cistus clusii Dunal	3	White	Sessile	Linear	Oligosperm
Cistus libanotis L.	3	White	Sessile	Linear-elliptical to linear	Oligosperm
Cistus munbyi Pomel	3	White	Sessile	Linear-lanceolate	-

Appendix 2. *(Continued)*

	Stigma level (style length in mm)	**No. of stamens**	**Exine thickness** (μm)	**Exine sculpturing**	**No. of fruit valves**
Subgenus *Cistus*					
Cistus albidus L.	Similar to stamens (2.5-5)	± 100	1.4	Rugulose	(4)5
Cistus chinamadensis Bañares et Romero	Above stamens (7-12)	-	-	-	5
Cistus creticus L.	Similar to stamens (2-5)	± 120	1.4	Rugulose	5
Cistus crispus L.	Similar to stamens (2-5)	± 80	1.4	Rugulose	5
Cistus heterophyllus Desf.	Similar to stamens (2-5)	± 150	1.4	Rugulose	5
Cistus ochreatus C. Sm. ex Buch	Above stamens (6-10)	± 200	-	-	5
Cistus osbeckiifolius Webb ex Christ	Above stamens (6-10)	-	-	-	5
Cistus parviflorus Lam.	Sessile (0)	± 60	-	Rugulose	5
Cistus symphytifolius Lam.	Above stamens (10-20)	± 150	-	-	5
Subgenus *Leucocistus* Willk.					
Cistus ladanifer L. ssp. *africanus* Dans.	Sessile (0)	± 150	4.2	Reticulate	6-10
Cistus ladanifer L. ssp. *ladanifer*	Sessile (0)	± 150	4.2	Reticulate	(7)9-10(12)
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts.	Sessile (0)	± 150	4.2	Reticulate	(6)8-9(10)
Cistus laurifolius L.	Subsessile (<0.5)	± 120	4.2	Reticulate	(4)5
Cistus monspeliensis L.	Below stamens (c. 0.5)	± 70	4.2	Retipilate	5
Cistus populifolius L. ssp. *populifolius*	Sessile (0)	± 100-120	4.2	Reticulate	5
Cistus populifolius L. ssp. *major* (Dunal) Heywood	Sessile (0)	± 100-120	4.2	Reticulate	5
Cistus pouzolzii Delile	Similar to stamens (2-5)	± 50	-	-	5
Cistus psilosepalus Sweet	Below stamens (c. 0.5)	± 150	4.2	Retipilate	5
Cistus salviifolius L.	Sessile (0)	± 100-120	4.2	Retipilate	5
Cistus albanicus E.F. Warb. ex Heywood	Subsessile (<0.5)	-	-	-	5
Subgenus *Halimioides* (Willk.) Demoly & P. Monts.					
Cistus clusii Dunal	Below stamens (1-2)	-	2.8	Striate	5
Cistus libanotis L.	Below stamens (c. 1)	-	2.8	Striate	5
Cistus munbyi Pomel	Below stamens (1-2)	-	2.8	Striate	5

4

Adaptive radiation in Mediterranean white-flowered *Cistus*

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Abstract

The *Cistus-Halimium* complex consists of 28 species distributed in the Mediterranean Basin and the Canary Islands. We have conducted "total evidence" analyses combining nuclear (ncp*GS*, ITS) and plastid (*trnL-trnF, trnK-matK, trnS-trnG, rbcL*) DNA sequences and using MP, ML and BI to infer a phylogenetic hypothesis of the group. Absolute divergence times of nodes are also estimated using a penalized likelihood approach. The *Cistus-Halimium* complex is formed by five well-supported lineages. One of them, the white-flowered *Cistus* lineage, comprises the higher number of species (12) and is monophyletic. This work supports a key tenet of the ecological theory of adaptive radiations not only for common ancestry, but also for rapid speciation, phenotype-environment correlation and trait utility. Molecular dating estimates a Mid Pleistocene (1.04 ± 0.25 Ma) diversification of the white-flowered lineage into two groups (*C. clusii* and *C. libanotis* lineages) with asymmetric number of species (2 vs. 10), similar vs. disparate leaf morphologies, floral characteristics and ecological attributes. Rapid speciation is also manifested by short branch lengths and fast speciation rates (2.13–3.49 species per Myr) in the *C. libanotis* lineage. A positive phenotype-environment correlation has been detected by historical reconstructions of morphological traits. MacClade transformation optimization and BayesTraits analysis of trait evolution are congruent with a significant correlated evolution between leaf shape and environment, leaf labdanum content and environment, and leaf pubescence and insolation conditions. These character traits are not associated with recent lineage splits in some sister species (but not in others), while phylogenetically separate sublineages meet comparable traits in typically Mediterranean environments. Trait utility is herein not explicitly tested. Circumstantial evidence indicates that modifications of leaf shape and size coupled with increases in labdanum secretion and pubescence density appear to be related to species success in different habitats. For instances, within the lineage species found in rather dense woods have more glabre, wider and larger leaves (e.g., *C. populifolius, C. laurifolius*) compared with those stickier and linear-lanceolate ones found in more xeric environments (e.g., *C. ladanifer, C. monspeliensis*). This study reports for the first time adaptive radiation documented for a Mediterranean plant group.

Key words: Adaptive radiation, labdanum, leaf shape, mainland, pubescence, trait utility

1. Introduction

The concept of adaptive radiation implies a rapid ecological diversification, which should be reflected in a greater morphological and/or physiological divergence among species in brief periods of rapid diversification from a single ancestor (Schluter 2000). Two mechanisms could generate adaptive radiations: (1) extrinsic causes due to new environmental circumstances (Baldwin & Robichaux 1995; Meimberg *et al.* 2006); (2) intrinsic characters of organisms (key innovation) that allow a taxon to utilize existing niche space in a novel manner (Hodges 1997). Remoteness and the rich diversity of habitats of island systems help ensure little competition and different environment to test the potential of plant radiations. In contrast to the wealth of studies documenting adaptive radiations on oceanic island (see, Baldwin & Robichaux 1995; Givnish *et al.* 1996; Kim *et al.* 1996) and particular mainland habitats (see, Reinthal & Meyer 1997; Givnish *et al.* 2000; Hughes *et al.* 2002), we have found in literature no study focused on the Mediterranean region.

The Mediterranean climatic type, characterized by a strong seasonality (hot dry summers, cool wet winters; Daget 1977; Nahal 1981), occurs in California, South Africa, central Chile, southern Australia, and typically in the Mediterranean Basin. In all five of these areas the native vegetation is a dense scrub characterized by winter annuals, drought deciduous, semi-deciduous malacophyllous species and woody evergreen sclerophyllous (Mooney & Dunn 1970). Sclerophyllous species are adapted to the low water availability during summer by means of showing small, leathery and dark leaves covered with thick cuticles and small, thick-walled cells (Schimper 1903; Read & Sanson 2003). Sclerophylly is so successful that unrelated genera converged into similar leaf traits.

Significant shrub components in the European-African Mediterranean ecosystems (e.g., "maquis", "garrigue") belong to the Cistaceae (*Halimium*, *Cistus*). *Cistus* is a genus of 21 frutescent and suffrutescent shrub species (Arrington & Kubitzki 2003) with a predominantly Mediterranean distribution, except five species endemic to the Canary Islands. Previous phylogenetic studies revealed the separation of the *Cistus-Halimium*

complex in two major natural groups: one of purple-flowered *Cistus* species (hereafter the purple-flowered lineage) and other containing the white-flowered *Cistus* and *Halimium* species, plus the pinkish *C. parviflorus* (hereafter the white-flowered lineage) (Guzmán & Vargas 2005). Leaf trichomes density, size, shape and tissue thickness (properties that influence the resistance to drought stress and solar irradiance) show marked variation across the white-flowered *Cistus*. Ecological results (Givnish 1984; Cunningham *et al.* 1999) analysing the role of changes in leaf morphological and physiological characters in dry environments appear to be related to radiation of the white-flowered *Cistus*.

In this study, we used DNA sequences data, sampled from both the nuclear (ITS, ncp*GS*) and the plastid (*trnL-trnF, trnK-matK, trnS-trnG, rbcL*) genomes to test explicit hypotheses of adaptive radiation by means of molecular phylogenetics in the *Cistus-Halimium* complex. Single ancestry and differentiation in short periods of time were first explored by phylogenetic and molecular clock analyses (Skelton 1993; Schluter 2000). Phenotype-environment correlation and trait utility analysis were further conducted to infer ecological characteristics (Robichaux *et al.* 1990; Givnish *et al.* 1995; Lowrey 1995) involved in speciation of *Cistus-Halimium* lineages.

2. Material and Methods

2.1. Sample strategy and DNA sequencing

A total of 35 individuals representing the 21 species of *Cistus*, one of *Fumana*, eight of *Halimium*, one of *Helianthemum* and one of *Tuberaria* were sampled for four plastid (*trnL-trnF* spacer, *trnS-trnG* spacer, *trnK-matK* spacer, *rbcL* exon) and two nuclear (*ITS*, ncp*GS*) DNA regions sequencing (Table 1; Appendix 1). Standard primers were used for amplification of the ITS region (White *et al.* 1990 for 17SE; Sun *et al.* 1994 for ITS4), the *trnL*(UAA)-*trnF*(GAA) (Taberlet *et al.* 1991), the *trnK-matK* (*trnK*-3914F and *matK*-1470R, Johnson & Soltis 1994) and the *trnS* (GCU)-*trnG* (UCC) (Hamilton 1999) spacers. The *rbcL* exon was amplified in two overlapping segments using the following primer combination: 1F-724R and 636F-1460R (Savolainen *et al.* 2000). A portion of the

glutamine synthetase (ncpGS) was amplified in 11 *Cistus* species with the universal primers Gscp687f and Gscp856r (Emshwiller & Doyle 1999). To ensure a homogeneous amplification reaction we design two 24-nucleotide-long primers specific for amplifying and sequencing *Cistus* species (CIS-687f: 5'GTAGCTGGAATCAACATCAGTGG3', CIS-856r: 5'GCTTGTTCAGTGATTCTCTGTCAG3').

Table 1. List of species used in the phylogenetic analysis

Taxon	*Locality/source*	*Voucher*
Cistus L.		
Cistus albanicus E.F. Warb. ex Heywood	Cultivated	R. G. Page 8cBGA04 (MA)P.
Cistus albidus L	Spain, Madrid, Aldea del Fresno	Vargas 25PV03 (MA)
Cistus chinamadensis Bañares et Romero	Canary Islands, La Gomera	Á. Fernández & J. Leralta 44BGA04 (MA)
Cistus clusii Dunal subsp. *clusii*	Spain, Málaga, Mijas	R. G. Page 8bBGA04 (MA)
Cistus clusii Dunal subsp. *multiflorus* Demoly	Spain, Balear Islands, Mallorca, Sa Rápita	C. Navarro *et al.* (618671MA)
Cistus creticus L.	Greece, Olympus	P. Vargas 209PV04 (MA)
Cistus crispus L.	Spain, Córdoba, Posadas	B. Guzmán 58BGA04 (MA)
Cistus heterophyllus Desf.	Morocco, Beni-Hadifa	B. Guzmán 99BGA04 (MA)
Cistus horrens Demoly	Canary Islands, Gran Canaria, Ayacata	B.Guzmán 2BGA05 (MA)
Cistus ladanifer L. subsp. *africanus*	Morocco, Targuist	B. Guzmán 109BGA04 (MA)
Cistus ladanifer L. subsp. *Ladanifer*	Spain, Madrid, Boadilla del Monte	B. Guzmán 7BGA03 (MA)
Cistus ladanifer L. subsp. *Sulcatus*	Portugal, Sagres	B. Guzmán 29BGA04 (MA)
Cistus laurifolius L.	Spain, Jaén, Sierra de Segura	B. Guzmán 13BGA03 (MA)
Cistus libanotis L.	Spain, Córdoba	R. G. Page 149BGA04 (MA)
Cistus monspeliensis L.	Portugal, Sagres	B. Guzmán 35BGA04 (MA)
Cistus munbyi Pomel	Morocco	O. Filippi 4BGA04 (MA)
Cistus ochreatus C. Sm. ex Buch	Canary Islands, Gran Canaria	R. G. Page 8BGA04 (MA)
Cistus osbeckiifolius Webb ex Christ	Canary Islands, Tenerife	P. Escobar 48/05 (MA)
Cistus parviflorus Lam.	Greece, Crete	O. Filippi 6BGA04 (MA)
Cistus populifolius L. subsp. *major* (Dunal) Heywood	Portugal, Ourique	B. Guzmán 20BGA04 (MA)
Cistus populifolius L. subsp. *populifolius*	Spain, Ávila, Arenas de San Pedro	P. Vargas 5PV03 (MA)
Cistus pouzolzii Delile	France	R. G. Page 8tBGA04 (MA)
Cistus psilosepalus Sweet	Spain, Ávila, Arenas de San Pedro	P. Vargas 7PV03 (MA)
Cistus salviifolius L.	Spain, Ávila, Arenas de San Pedro	P. Vargas 6PV03 (MA)
Cistus symphytifolius Lam.	Canary Islands, La Palma, La Cumbrecita	B. Guzmán 143BGA04 (MA)
Fumana (Dunal) Spach		
Fumana thymifolia (L.) Spach ex Webb	Portugal, Ferrerías	B. Guzmán 53BGA04 (MA)
Halimium (Dunal) Spach		
Halimium atlanticum Humbert & Maire	Morocco, Tazzeka	RDG14/2006/5
Halimium atriplicifolium (Lam.) Spach	Spain, Granada, Sierra Nevada	P. Vargas 120PV04 (MA)
Halimium calycinum (L.) K. Koch	Portugal, Cabo Sardao	B. Guzmán 49BGA04 (MA)
Halimium halimifolium (L.) Willk. *Halimifolium*	Spain, Málaga, Marbella	A. Segura (580185MA)
Halimium lasiocalicynum (Boiss. & Reut.) Gross ex Engl. subsp. *riphaeum* (Pau & Font Quer) Maire	Morocco, Bab-Berred	P. Escobar 665/04 (MA)
Halmium ocymoides (Lam.) Willk.	Portugal, Coimbra	R. G. Page 158BGA04 (MA)
Halimium umbellatum (L.) Spach	Spain, Madrid, Tres Cantos	P. Vargas 71BGA04 (MA)
Helianthemum Mill.		
Helianthemum squamatum (L.) Dum. Cours.	Cultivated	B. Guzmán 70BGA04 (MA)
Tuberaria Dunal		
Tuberaria guttata (L.) Fourr.	Portugal, Vila do Vispo	B. Guzmán 44BGA04 (MA)

After 1-3 min pretreatment at 94 °C, PCR conditions for amplification were: 24-39 cycles of 1 min at 94 °C, 30 s-1 min at 48-50-55 °C and 1-4 min at 72 °C. A volume of 1 μL

of dimethyl-sulfoxide (DMSO) was included in each 25 µl reaction. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism model 3700 automated sequencer. PCR primers were used for cycle sequencing of the spacers, the *rbcL* exon and the ncp*GS* gene while the ITS 5 and ITS 4 (Sun *et al.* 1994) primers were used for cycle sequencing the ITS region. Additionally, due to mononucleotide repeat stretches (poly-T, poly-A) the internal primer trnSGpolyTf (Guzmán & Vargas, unpublished) was used to sequence the *trnS-trnG* spacer in the purple-flowered species. Sequenced data were assembled and edited using the program Seqed (Applied Biosystems, California). The limits of the regions were determined by position of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

2.2. *Molecular analyses*

2.2.1 Phylogenetic analyses

Maximum Parsimony (MP) and Bayesian Inference (BI) analyses were performed on one combined molecular data set (*trnL-trnF, trnS-trnG, trnK-matK, rbcL, ITS,* ncp*GS*). Sequences were aligned using Clustal X 1.62b (Thompson *et al.* 1997), with further adjustments by visual inspection. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford 1999) with equal weighting of all characters and of transitions/transversions. Heuristic searches were replicated 1000 times with random taxon–addition sequences, tree–bisection–reconnection (TBR) branch swapping, the options MulTrees and Steepest Descent in effect and holding 10 trees per replicate. Internal support was assessed using 5,000,000 bootstrap replicates (fast stepwise-addition, Mort *et al.* 2000).

To determine the simplest model of sequence evolution that best fits the sequence data, the Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion

(AIC) were implemented using MrModeltest 1.1b (Posada & Crandall 1998; Nylander 2002) in each data set. A Bayesian Inference analysis (BI) was conducted in MrBayes 3.0b4 (Ronquist & Huelsenbeck 2003) using two identical searches with two million generations each (four MCMC, chain temperature=0.2; sample frequency=100). In both runs probabilities converged at the same stable value after generation 100,000 approximately. A 50% majority–rule consensus tree was calculated using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro *et al.* 2003). Branches with parsimony bootstrap (BS) values above 85% and posterior probabilities (PP) above 95 were considered to have strong support, and branches with BS values between 71% and 84% and PP between 80 and 94 were considered to have moderate support. Branches with lower values were considered to have weak support.

2.2.2. Character evolution

Patterns of evolution of six key traits (leaf shape, leaf labdanum secretion, leaf pubescence, soil requirements, insolation conditions, environment) were explored using MacClade 4.06 (Maddison & Maddison 1992) assuming Fitch Parsimony and treating characters as unordered.

Additionally, to account for values of phylogenetic and mapping uncertainty, probabilities of ancestral states for the six traits were estimated individually using the BayesMultiState program, contained in the BayesTraits 1.0 package (Pagel & Meade 2007), under MCMC method and allowing transitions between character states in both directions. To reduce the autocorrelation of successive samples, 1000 trees were drawn from the distribution of 1.9×10^6 trees, which equates to sampling every 1900th generation of the chains used in the phylogenetic analysis. As suggested in BayesMultiState manual, to reduce some of the uncertainty and arbitrariness of choosing prior in MCMC studies, we used the hyperprior approach, in concrete the reversible-jump (RJ) hyperprior with a gamma prior (mean and variance seeded from uniform distributions on the interval 0 to 10). Preliminary analyses were run to adjust

the *ratedev* parameter until the acceptance rates of proposed changes was around 20-40%. Using *ratedev* settings (Appendix 2), we ran the RJ MCMC analyses for each trait three times independently for 1.0 x 10^7 iterations, sampling every hundredth iteration (to produce 90,000 sampled points) and discarding the first 1,000,000 iterations. All runs gave mostly the same results and we report one of them here. We use the "Addnode" command to find the proportion of the likelihood associated with each of the possible states at each node.

2.2.3. Testing correlated evolution

We modelled correlated evolution of discrete binary traits (leaf shape/insolation conditions, leaf shape/habit, labdanum secretion/insolation conditions, labdanum secretion /habit, leaf pubescence/insolation conditions, leaf pubescence/habit) on 1000 bayesian trees using the BayesDiscrete program, contained in the BayesTraits 1.0 package (Pagel & Meade 2007), and the same parameters described above. The method compares the statistical likelihood of a model in which two binary traits are allowed to evolve independently on the tree, with a model in which the two traits are allowed to evolve in a correlated fashion. Evidence for correlated evolution arises if the dependent or correlated model shows significantly better fit to the data than the independent model. As the independent and dependent models are estimated by MCMC, their goodness of fit is compared using the log-Bayes Factor test: 2*log[harmonic mean(dependen model)] – log[harmonic mean (independent model)].

As binary traits are required we coded traits as followed: leaf shape, 0 linear to elliptic, 1 ovate-lanceolate to ovate; labdanum secretion, 0 zero to eight percent, 1 nine to fifteen; upper leaf pubescence, 0 glabre to subglabre, 1 dense tomentum; insolation conditions, 0 helioxerophyllous to subheliophyllous, 1 subsciophyllous to submesophyllous; environment, 0 bushy and scrub vegetation, 1 woodlands.

2.2.4. Haplotype data analysis

Sequences of plastid DNA (*trnL-trnF, trnK-matK trnS-trnG* and *rbcL*) were combined to analyse relationships among the white-flowered *Cistus* (plus *C. parviflorus*) plastid haplotypes. We used the software TCS 1.21 (Clement *et al.* 2000). The program implements a statistical parsimony approach using the algorithm described in Templeton *et al.* (1992) to construct haplotype networks. The maximum number of differences among haplotypes, as a result of single substitutions, was calculated with 95% confidence limits and treating gaps as missing data.

2.2.5. Molecular dating

Divergence dates were estimated for nodes of the Bayesian consensus tree. To check the constancy of substitution rates we used the Langley and Fitch (LF) test (Magallón & Sanderson 2005). We rejected the null hypothesis of constant rate (χ^2=5204.26; d.f.=34) and, then, divergence times were estimated using the r8S 1.71 program (Sanderson 2002) with a Penalized Likelihood (PL) approach. Penalized Likelihood was implemented with the Truncated Newton (TN) algorithm. Initial results were obtained under the following parameters: cvstart=0.5; cvinc=0.5; cvnum=10 with cross-validation enforced to estimate the rate smoothing parameter (measure of the rate variation and autocorrelation of rates from clade to clade). The rate smoothing with the lowest crossvalidation score was selected and the dating procedure was repeated with the following parameters: collapse; num_time_guesses=5 and num_restarts=5. Crossvalidation suggested 10 as the best smooth parameter. Branching order and branch lengths from 100 Bayesian trees sampled every 10,000 generations after stationary were analyzed to obtain means and standard deviations of clade ages (Hughes & Eastwood 2006). To convert relative divergence times into absolute time units we used two maximum-age fossil constraints. Palynological studies identified *Helianthemum* pollen in Upper Miocene formations (11 Ma) from France (Naud & Suc 1975) and *Tuberaria* pollen in Pliocene formations (5.3 Ma) from Germany (Menke 1976).

Species diversification rates, assuming an equal rate of random speciation Yule model, were calculated using the formula $SR=[(\log_e(N)-\log_e(N_0)]/T$ (Kendall 1949; Moran 2000; Hughes & Eastwood 2006), where N is the total number of extant species in the clade of interest, N_0 is the initial species diversity, usually taken as 1, and T is the inferred age of the clade (million years). Upper and lower standard deviations of age estimates were used in calculations of speciation rates.

3. Results

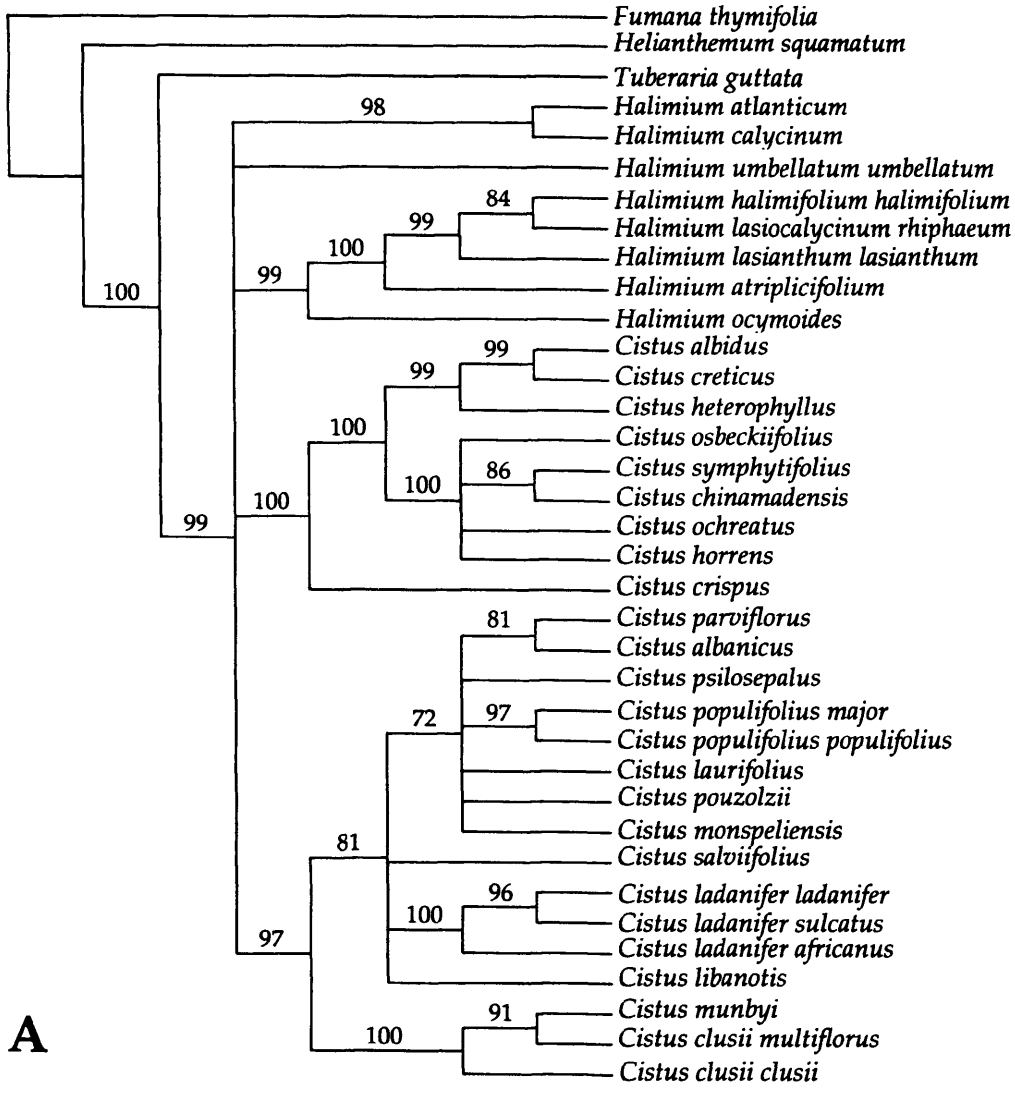
3.1. Phylogenetic analyses

The characteristics of the six data sets are summarized in Table 2. MP analysis using Fitch parsimony resulted in 104 shortest trees of length 1317 steps. The consistency

Table 2. Statistics for each of the DNA sequence regions used in the phylogenetic analysis of the *Cistus-Halimium* complex

	trnS-trnG	*trnL-trnF*	*trnK-matK*	*rbcL*	ITS	ncp*GS*
Cistaceae						
Length (bp)						
Total aligned length	1084	516	1403	1404	697	402
Length range - ingroup	617-824	399-461	1302-1357	1403-1404	644-650	340-452
Length range - outgroup	158-684	377-422	1301-13016	1404	585-654	318
Number of characters						
Total included	713	516	1403	1379	697	402
Variable/parsimony-informative	148/54	128/52	280/108	103/44	203-69	86/17
Mean G+C content	21%	33%	33%	43%	65%	40%
Maximum sequence divergence (GTR)	17.92%	14.1%	14.08%	4.11%	20.37%	35.4%
Sequence evolution model (Akaike Test)	GTR+G	GTR+G	GTR+G	GTR+I	GTR+I+G	HKY+G
White-flowered *Cistus* plus *C. parviflorus*						
No. of variable/parsimony-informative characters	45/25	28/11	33/12	20/10	75/33	25/8
Maximum sequence divergence (GTR)	1.90%	3.15%	0.85%	0.74%	4.21%	3.11%
Sequence evolution model (Akaike Test)	GTR+I	F81+I	GTR	HKY	HKY+I+G	HKY+G

index (CI) for these trees was 0.82 and the retention index (RI) was 0.80. Several strongly supported clades were present (Fig. 1). Analyses provided strong (99% BS, 94 PP) support for the monophyly of the *Cistus-Halimium* complex. Parsimony and



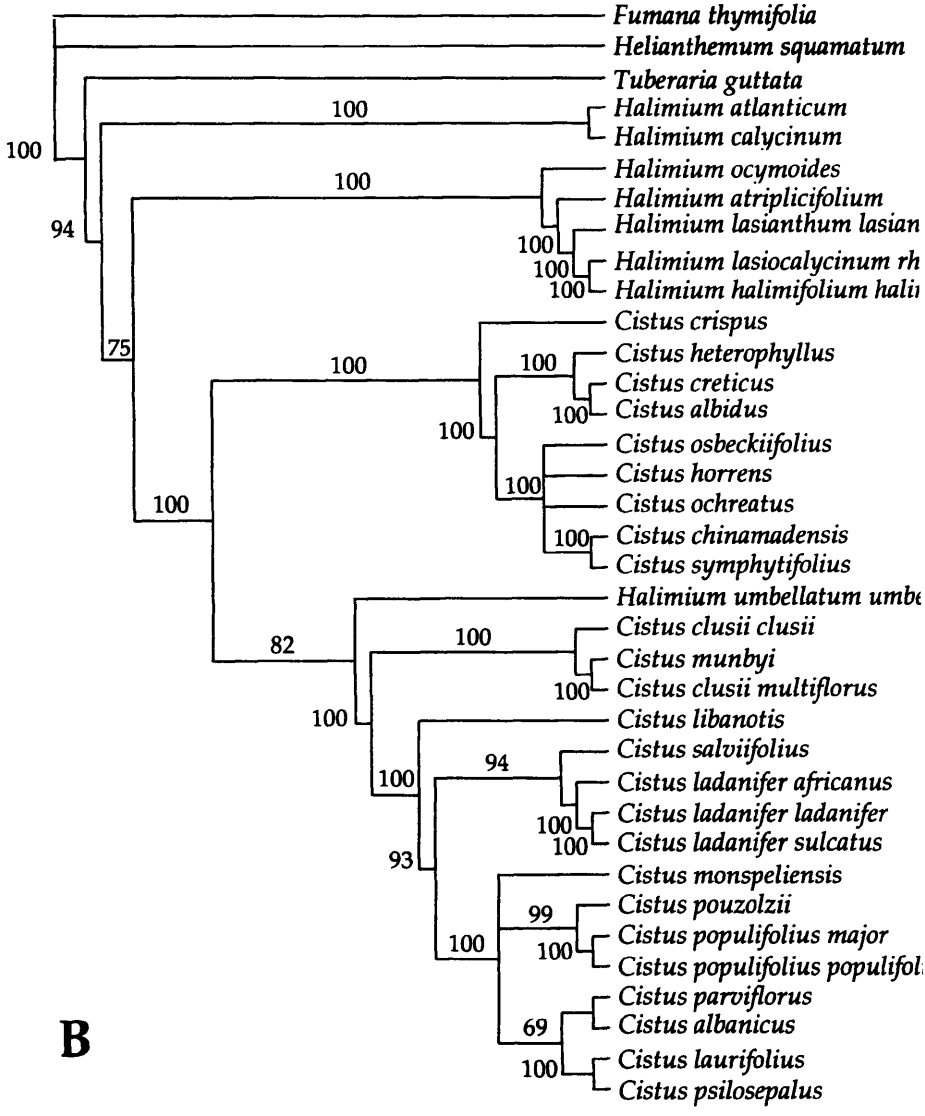


Fig. 1. Phylogenetic hypothesis based on plastid (*trnL-F, trnK-matK, trnS-trnG, rbcL)* and nuclear (ITS, ncp*GS)* sequences. (A) Strict consensus of 1 equally parsimonious trees of 1317 steps (CI = 0.82, RI = 0.80, RC = 0.66), showing bootstrap support for clades above branches; (B) Bayesian inferen tree (50% majority rule consensus tree) showing posterior probabilities above branches.

Bayesian consensus trees were consistent at different places: (1) *Cistus* species are not monophyletic; (2) *Cistus* species are divided in two lineages, one of purple-flowered species (except *C. parviflorus*) (100% BS, 100 PP) and other of white-flowered species plus *C. parviflorus* (97% BS, 100 PP); (3) *Cistus crispus* is the sister-group of the rest of purple-flowered species (100% BS, 100 PP) and a sister-group relationship exists between the *C. clusii-C. munbyi* lineage and the rest of the white-flowered species plus *C. parviflorus* (81% BS, 100 PP).

3.2. Evaluating patterns of trait evolution

The range of interspecific variation in leaf morphology and ecological requirements is shown in Table 3. Character reconstruction of three morphological and three ecological characters are mapped on the Bayesian consensus tree (Fig. 2) to investigate patterns of evolution. The most relevant results from the historical reconstructions are following described:

1. Leaf shape (Fig. 2A). The character state reconstruction shows linear leaves as a plesiomorphic state. Ovate-lanceolate and ovate shapes evolved twice in the *C. libanotis* lineage, while only one event was necessary to originate linear-lanceolate to elliptic leaves.

2. Labdanum secretion (Fig. 2B). The character is equivocal in most of the *C. libanotis* lineage because, in part, of missing data from two species. A medium percentage of secretion per unit leaf dry weight (5-10) is however traced as the ancestral state.

3. Upper leaf pubescence (Fig. 2C). The character is revealed as very homoplastic within the *C. libanotis* lineage. Despite the reconstruction is equivocal tracing the state of some nodes, independent acquisition (up to three times) of a dense tomentum can be suggested.

4. Soil (Fig. 2D). The historical reconstruction traces silicolous soils as the ancestral state. It is noteworthy that the only two species inhabiting basic and ultrabasic soils within the *C. libanotis* lineage belong to the same sublineage.

Table 3. Morphological and environmental characteristics of the white-flowered *Cistus* lineage. Data were taken from Grosser (1903), Martín & Guinea (1949), Dansereau (1958)*, Warburg (1968), Demoly and Montserrat (1993), Greuter (1996), Gülz *et al.* (1996)** and own observations

	Soil	Climate conditions	Altitude (m)	Insolation conditions*, environment	Leaf shape (length x width in mm)
C. albanicus	serpentin	mesic, mountain Mediterranean	1000-1500	submesophyllous, *Abies cephalonica* woodlands	elliptic (3-5 x 0.8-1.5)[1]
C. clusii	calcicolous	dry to semi-arid, coast Mediterranean	0-1500	helioxerophyllous, bushy vegetation	linear (10-26 x 1-2)
C. ladanifer	silicolous	dry, Mediterranean	0-1500	subheliophyllous, degradated *Quercus suber/ilex* woodlands	linear-lanceolate (40-80 x 6-21)
C. laurifolius	silicolous	mesic, mountain Mediterranean	1900 (400-2800)	submesophyllous, degradated *Q. pyrenaica/faginea* and *Pinus* woodland	ovate-lanceolate (40-90 x 17-30)
C. libanotis	silicolous, sandy	dry, coastal Mediterranean	0-100	subsciophyllous, degradated *Pinus halepensis/pinea* and *Quercus suber* woodlands	linear (22-40 x 2-5)
C. monspeliensis	silicolous	dry, Mediterranean	0-1200	subheliophyllous, degradated *Quercus suber/ilex* and *Pinus* woodlands	linear-lanceolate (15-45 x 2-7)
C. munbyi	calcicolous	coastal mediterranean	0-100	helioxerophyllous, bushy vegetation	linear (6-30 x 1-4)
C. parviflorus	calcicolous	dry coastal Mediterranean	0-600	helioxerophyllous, scrub vegetation	ovate (15-30 x 7-27)
C. populifolius	silicolous	dry, Mediterranean	200-1500	submesophyllous, degradated *Quercus* and *Pinus* woodlands	ovate-lanceolate (50-95 x 25-55)
C. pouzolzii	silicolous	dry, mountain Mediterranean	800-1800	subheliophyllous, degradated *Quercus suber/ilex* and *Pinus* woodlands	lanceolate-elliptic (20-31 x 4-11)
C. psilosepalus	silicolous	humid, Atlantic influence	0-1100	submesophyllous, scrub vegetation	lanceolate-elliptic (30-65 x 10-23)
C. salviifolius	silicolous/ calcicolous	humid to dry, Mediterranean and Eurosiberian	0-1800	subheliophyllous/submesophyllous, degradated woodlands of many types	ovate (8-18 x 7-12)

Table 3. *(Continued)*

	Leaf margin	Leaf venation	Leaf surface and texture	Labdanum secretion**,[2]	Leaf non-secretorial trichomes**	
					Upper surface	Lower surface
C. albanicus	flat	reticulate	smooth, soft	1.0	long single, stellate	glabre
C. clusii	revolute	uni-nerve	smooth, coriaceous	6.0	subglabre with tuft of single hairs	dense tomentum of stellate
C. ladanifer	flat	pinnate	smooth, coriaceous	12.5	glabre	dense tomentum of stellate
C. laurifolius	slightly crispate	parallel	smooth, coriaceous	13.5	glabre	dense tomentum of single and stellate (deciduous)
C. libanotis	revolute	uni-nerve	smooth, coriaceous	6.1	subglabre, stellate	dense tomentum of stellate
C. monspeliensis	flat, slightly revolute	parallel	smooth, coriaceous	10.7	single and scarce	dense tomentum of minute stellate
C. munbyi	revolute	uni-nerve	smooth, coriaceous	-	subglabre	dense tomentum of stellate
C. parviflorus	flat	parallel	smooth, coriaceous	1.2	dense tomentum of stellate	dense tomentum of stellate
C. populifolius	flat	pinnate	smooth, coriaceous	5.6	glabre	glabre
C. pouzolzii	crispate	parallel	rough, coriaceous	-	dense tomentum of single and stellate	dense tomentum of single and stellate
C. psilosepalus	flat	reticulate	smooth, soft	2.0	single and scarce	stellate
C. salviifolius	slightly crispate	pinnate	rough, coriaceous	0.5	stellate	stellate

[1] values from 16 leaves
[2] % per unit leaf dry weight

5. Insolation conditions (Fig. 2E). The optimization is equivocal reconstructing the ancestral state in the *C. libanotis* lineage. Two sister species groups underwent a dramatic change in insolation conditions (*C. parviflorus-C. albanicus; C. populifolius-C. pouzolzii*). Although ancestral character states are poorly optimised for insolation conditions, reversal to high solar exposure (helioxerophyllous) is retrieved in *C. parviflorus*.

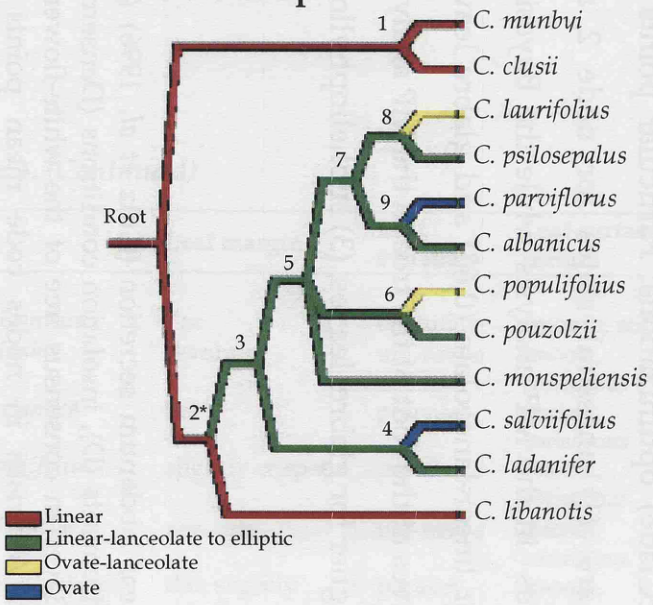
6. Environment (Fig. 2F). The frequency in habitat change is found in the *C. libanotis* lineage. Similar environments are shared in a few groups with (*C. pouzolzii, C. populifolius*) or without (*C. psilosepalus, C. parviflorus*) phylogenetic relationship. In contrast, four habitats are occupied by four species suggesting a dynamic habitat change in the course of evolution.

BayesTraits analysis of trait evolution was used to test reconstruction uncertainty. Appendix 2 reports ratedev settings and mean values (± 95% confidence intervals) of the log-likelihood and posterior distributions of the rate of coefficients obtained from the reversible jump (RJ) MCMC analysis. The mean of the Bayesian posterior probabilities of each character state at every node are provided in Table 4. The 95% confidence intervals of the posterior probabilities were all lower than ± 0.004. The Bayesian results mostly support MP (MacClade) optimizations. Particular points of disagreement between both approaches are: (1) ancestral states for node 2 are reconstructed as linear and subglabre leaves in the MP analysis while the Bayesian approach estimates a higher probability for linear-lanceolate (0.68) and glabre leaves (0.68) states to be ancestral; (2) subglabre leaves at the root of the tree in the MP analysis whereas the Bayesian probability (0.52) is higher for glabre leaves; (3) subheliophyllous

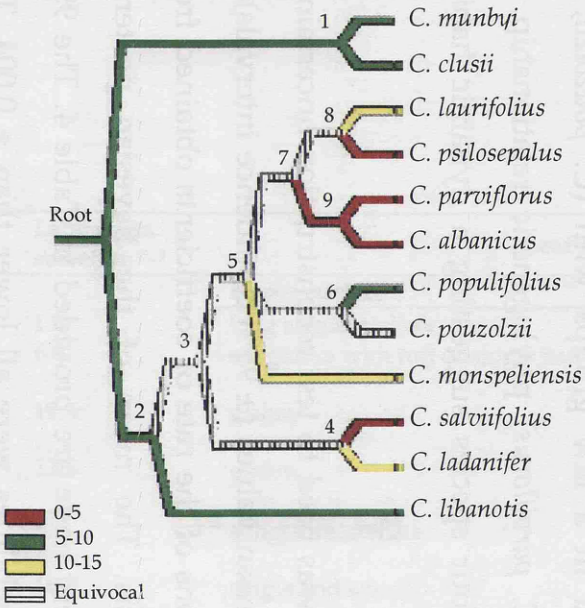
→

Fig. 2. Historical patterns of leaf shape (A), leaf labdanum secretion (Gülz *et al.* 1996) (B), pubescence of upper leaf surface (C), soil requirements (D), insolation conditions (Dansereau 1958) (E), environment (F) mapped onto the Bayesian consensus tree of the white-flowered *Cistus* lineage. Nodes code above branches. Asterisks in nodes code mean points of disagreement with the Bayesian approach of trait evolution.

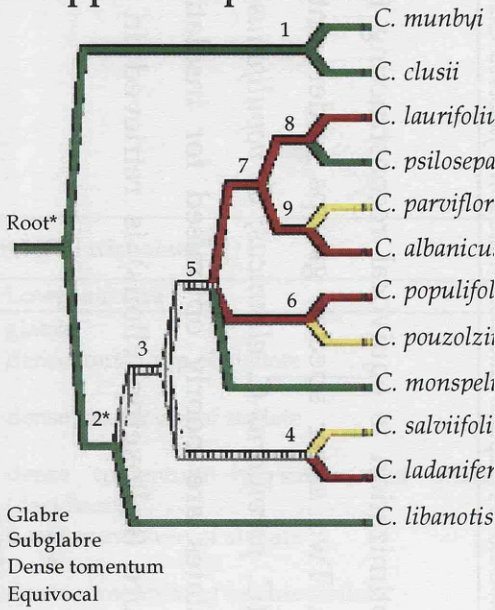
A. Leaf shape



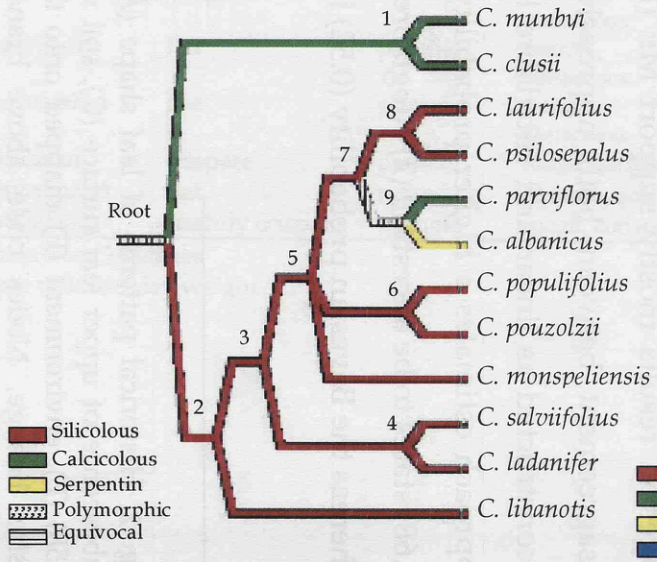
B. Labdanum secretion*



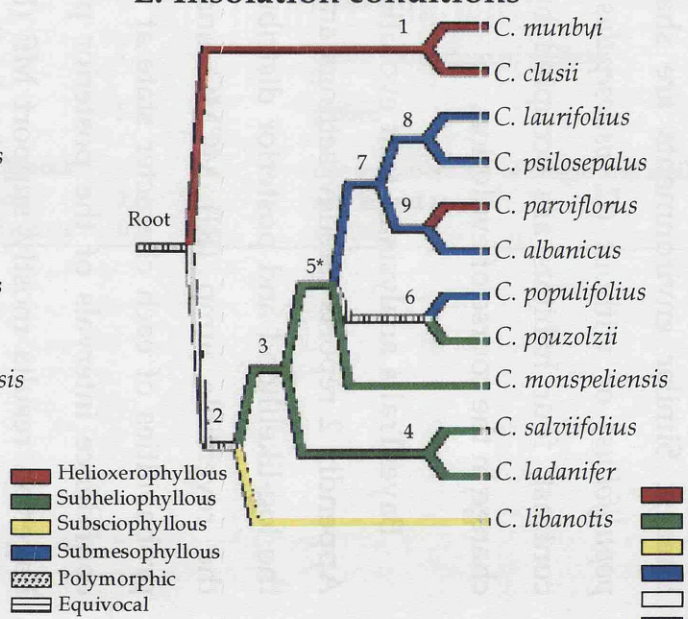
C. Upper leaf pubescence



D. Soil



E. Insolation conditions



F. Environment

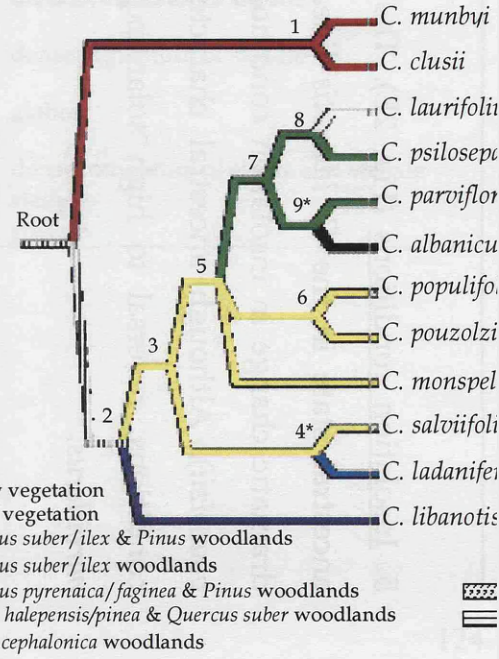


Table 4. Mean of posterior probabilities of Bayesian inference character state evolution of successive iterations (9,000,000) by RJ MCMC. The 95% confidence intervals of the posterior probabilities were all less than ± 0.004. In bold character state evolution as traced in MacClade (Maddison & Maddison 1992) (Fig. 2). Particular points of disagreement between Bayesian and the MacClade optimization approach are underlined. Node codes as in Fig. 2

	Leaf shape[a]	Labdanum secretion[b]	Leaf pubescence[c]	Soil[d]	Insolation conditions[e]	Environment[f]
Root	**0.42**/0.42/0.08/0.07	0.23/**0.49**/0.28	0.52/**0.36**/0.12	0.77/0.17/0.06	0.32/0.31/0.16/0.21	0.21/0.10/0.22/0.14/0.11/0.13/0.09
Node 1	**0.99**/0.00/0.01/0.00	0.05/**0.89**/0.06	0.01/**0.98**/0.01	0.00/**0.99**/0.01	**0.99**/0.00/0.00/0.01	**0.96**/0.01/0.01/0.01/0.01/0.00/0.00
Node 2	**0.18**/0.68/0.07/0.07	0.30/**0.34**/0.36	0.68/**0.19**/0.13	**0.98**/0.01/0.01	0.05/0.45/0.17/0.33	0.06/0.09/0.33/0.18/0.12/0.14/0.08
Node 3	0.03/**0.86**/0.06/0.05	0.35/0.22/0.43	0.75/0.10/0.15	**0.98**/0.01/0.01	0.02/**0.51**/0.02/0.45	0.06/0.09/**0.40**/0.19/0.12/0.06/0.08
Node 4	0.08/**0.60**/0.09/0.23	0.32/0.14/0.54	0.66/0.09/0.25	**0.89**/0.07/0.04	0.06/**0.76**/0.06/0.12	0.09/0.09/**0.13**/0.38/0.13/0.09/0.09
Node 5	0.02/**0.85**/0.11/0.02	0.38/0.31/0.31	0.70/0.15/0.15	**0.96**/0.02/0.02	0.02/**0.27**/0.01/0.70	0.04/0.11/**0.57**/0.04/0.12/0.04/0.08
Node 6	0.04/**0.60**/0.32/0.04	0.09/0.80/0.11	**0.58**/0.06/0.36	**0.98**/0.01/0.01	0.05/0.49/0.05/0.41	0.03/0.03/**0.74**/0.04/0.10/0.03/0.03
Node 7	0.04/**0.73**/0.15/0.08	0.65/0.09/0.26	**0.67**/0.21/0.12	**0.80**/0.08/0.12	0.01/0.02/0.01/**0.96**	0.07/**0.33**/0.07/0.08/0.18/0.08/0.19
Node 8	0.07/**0.55**/0.31/0.07	0.46/0.12/0.42	**0.53**/0.38/0.09	**0.95**/0.02/0.03	0.03/0.04/0.04/**0.89**	0.08/**0.33**/0.08/0.09/0.25/0.08/0.09
Node 9	0.07/**0.62**/0.08/0.23	**0.90**/0.05/0.05	**0.70**/0.07/0.23	0.11/0.31/0.58	0.03/0.04/0.04/**0.89**	0.08/**0.18**/0.08/0.08/0.08/0.08/0.42

Note: Values in the table reflect estimates based on the averaging over 1000 Bayesian tree

[a] Leaf shape: Linear/Linear-lanceolate to elliptic/Ovate-lanceolate/Ovate

[b] Labdanum secretion: 0-5/5-10/10-15 % per unit leaf dry weight

[c] Leaf pubescence: Glabre/Subglabre/Dense tomentum

[d] Soil: Silicolous/Calcicolous/Serpentin

[e] Insolation conditions: Helioxerophyllous/Subheliophyllous/Subsciophyllous/Submesophyllous

[f] Environment: Bush/Scrub/*Quercus suber-ilex* & *Pinus* woodlands/ *Quercus suber-ilex* woodlands/*Quercus pyrenaica-faginea* & *Pinus* woodlands/*Pinus halepensis-pinea* & *Quercus suber* woodlands/*Abies cephalonica* woodlands

condition is ancestral at node 5 in the MP optimization but the submesophyllous condition displays the highest posterior probability (0.70); (4) the historical reconstruction using the MP optimization traces *Quercus suber/ilex* & *Pinus* woodlands as the ancestral state at node 4 (*C. ladanifer-C. salviifolius* lineage) while *Quercus suber/ilex* woodlands show the highest posterior probability (0.38); (5) scrub vegetation is the ancestral state at node 9 (*C. parviflorus-C. albanicus* lineage) using the MacClade optimization, while *Abies cephalonica* woodlands display the highest posterior probability (0.42).

3.3. Bayesian analysis of correlated evolution

Table 5 shows the log-Bayes factor calculations and significance following the scale of Bayes factor test presented by Kass & Raftery (1995). The evolution of leaf traits was not closely associated with ancestral changes in environment and insolation conditions. There is evidence against a correlated evolution between insolation conditions and two leaf traits (leaf shape (log-Bayes factor = -1.5), labdanum secretion (log-Bayes factor = -1.8)). Additionally, barely evidence against correlated evolution for leaf pubescence/environment has been found (log-Bayes factor = -0.1). On the other hand, barely correlated evolution is suggested between the next pairs of variables: leaf shape/environment (log-Bayes factor = 0.7), labdanum secretion/environment (log-Bayes factor = 0.8), leaf pubescence/insolation conditions (log-Bayes factor = 0.6).

Table 5. Calculations for log-Bayes factor tests in favour of a dependent model. In the final column, we followed the Bayes factor test (Kass and Raftery (1995) in our interpretation of the log-Bayes factor.

	Log-harmonic mean[a]		log-Bayes factor	Significance
	Dependent model	Independent model		
Leaf shape/Environment	-12.72	-13.06	0.7	barely in favour
Leaf shape/Insolation	-18.12	-17.36	-1.5	against
Labdanum secretion/Environment	-11.99	-12.39	0.8	barely in favour
Labdanum secretion/Insolation	-17.23	-16.33	-1.8	against
Leaf pubescence/Environment	-11.49	-11.44	-0.1	barely against
Leaf pubescence/Insolation	-16.25	-16.58	0.6	barely in favour

[a] Mean calculated from 9,000,000 iterations values

3.4. Haplotype analysis of the white-flowered Cistus lineage

Sequence length of the white-flowered *Cistus* lineage was 417-461 bp for *trnL-trnF*, 561-585 for *tnrS-trnG*, 1309-1357 for *trnK-matK* and 1378-1379 for *rbcL* (Appendix 3). The combined data of the three spacers and the gene of 13 white flowered *Cistus* plus *C. parviflorus* taxa distinguished 12 haplotypes (Appendix 2). Haplotypes were exclusive to a single species or subspecies (Table 1). Only *C. ladanifer* subspp. *ladanifer* and *sulcatus* showed the same haplotype. TCS constructed a single network (Fig. 3) including 12 haplotypes and no loops. *Cistus populifolius* subsp. *populifolius* was depicted as the ancestral one.

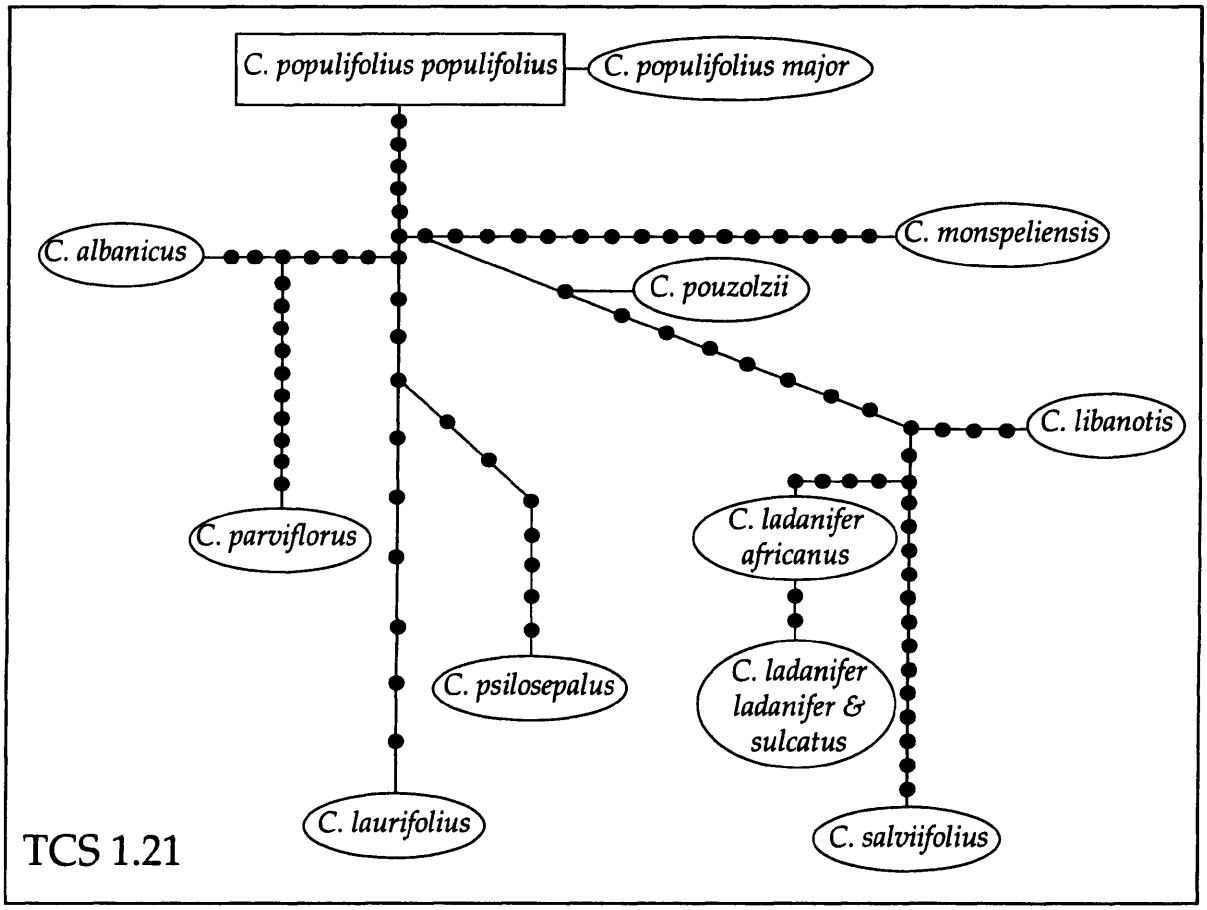


Fig. 3. Statistical parsimony network representing relationships of the 12 plastid (*trnL-trnF, trnK-matK, rbcL, trnS-trnG*) haplotypes of the white-flowered *Cistus* lineage. Lines indicate mutation steps (single nucleotide substitutions) and dots (•) represents missing haplotypes (extinct or not found).

3.5. Estimates of divergence times

Results of dating analysis are shown in Table 6 and Fig. 4. In general, the data indicate a later Pliocene 2.11 ± 0.87 Ma) divergence between *Tuberaria* and the *Cistus-Halimium* complex, followed by a Pleistocene differentiation of the major clades of the complex.

Table 6. Penalized Likelihood (bootstrapping of 100 trees) molecular clock estimates of ages for constrained and unconstrained nodes. Nodes A and B are assigned a maximum age (indicate in parentheses) as derived from palynological studies (Naud & Suc 1975; Menke 1976). Letters and numeric codes for each node of the phylogeny of Cistaceae correspond to those shown in Fig. 4. Ma = million years ago; SD = Standard deviation

Node	Mean age (Ma)	SD (Ma)	Maximum age (Ma)	Minimum age (Ma)
A (11)	9.65	2.21	11.00	0.58
B (5.3)	4.87	1.10	5.30	0.21
1	2.11	0.87	4.93	0.14
2	1.01	0.31	1.99	0.06
3	1.78	0.45	2.74	0.12
4	1.25	0.32	1.80	0.07
5	0.53	0.15	0.83	0.03
6	0.30	0.09	0.49	0.02
7	0.15	0.06	0.33	0.006
8	1.56	0.38	2.32	0.09
9	0.80	0.21	1.17	0.05
10	0.52	0.14	0.78	0.03
11	0.19	0.07	0.33	0.01
12	0.04	0.02	0.13	0.002
13	0.05	0.06	0.21	0.000
14	0.04	0.02	0.13	0.003
15	1.47	0.35	2.09	0.08
16	1.04	0.25	1.41	0.06
17	0.23	0.09	0.43	0.01
18	0.09	0.04	0.20	0.003
19	0.88	0.22	1.22	0.06
20	0.82	0.20	1.13	0.05
21	0.72	0.18	0.97	0.04
22	0.17	0.06	0.34	0.009
23	0.04	0.02	0.11	0.0003
24	0.65	0.16	0.89	0.04
25	0.45	0.12	0.71	0.02
26	0.06	0.04	0.21	0.002
27	0.61	0.23	0.91	0.00
28	0.31	0.23	0.67	0.00
29	0.28	0.28	0.71	0.00

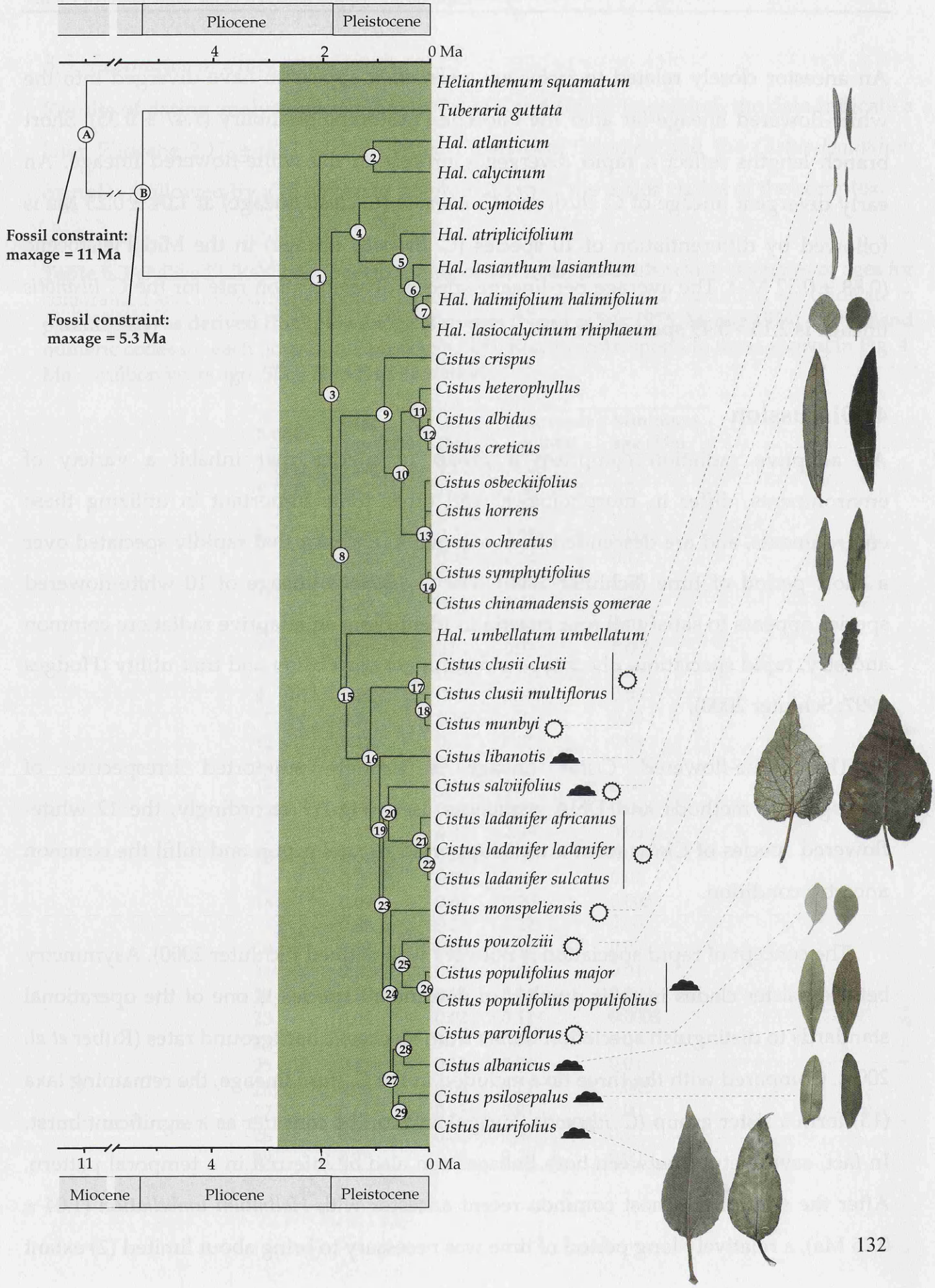
An ancestor closely related to *Halimium umbellatum* appear to have diverged into the white-flowered lineage far after the Pliocene-Pleistocene boundary (1.47 ± 0.35). Short branch lengths reflect a rapid divergence process in the white-flowered lineage. An early divergent lineage of *C. clusii* and *C. mumbyi* (*C. clusii* lineage) at 1.04 ± 0.25 Ma is followed by differentiation of 10 species (*C. libanotis* lineage) in the Mid Pleistocene (0.88 ± 0.22 Ma). The average per-lineage species diversification rate for the *C. libanotis* lineage is 2.13 – 3.49 species per Myr.

4. Discussion

An adaptive radiation comprises a group of species that inhabit a variety of environments, differ in morphological and other traits important in utilizing these environments, and are descended from a common ancestor that rapidly speciated over a short period of time (Schluter 2000). The *C. libanotis* lineage of 10 white-flowered species appears to satisfy all four criteria as identifying an adaptive radiation: common ancestry, rapid speciation, phenotype-environment correlation and trait utility (Hodges 1997; Schluter 2000).

The white-flowered *Cistus* lineage is strongly supported irrespective of phylogenetic methods and DNA sequences used (Fig.1). Accordingly, the 12 white-flowered species of *Cistus* form a well-supported natural group and fulfil the common ancestry condition.

The concept of rapid speciation is not very well defined (Schluter 2000). Asymmetry between sister clades in their number of descendant species is one of the operational standards to distinguish speciation bursts from stochastic background rates (Rüber *et al.* 2003). Compared with the three taxa included in the *C. clusii* lineage, the remaining taxa (13) form a sister group (*C. libanotis* lineage) and can be consider as a significant burst. In fact, asymmetries between both lineages can also be inferred in a temporal pattern. After the split of the most common recent ancestor with *Halimium umbellatum* (1.04 ± 0.25 Ma), a relatively long period of time was necessary to bring about limited (2) extant


Pliocene
Pleistocene
4
2
0 Ma
Helianthemum squamatum
Tuberaria guttata
A
B
Hal. atlanticum
2
Hal. calycinum
Hal. ocymoides
4
Hal. atriplicifolium
5
Hal. lasianthum lasianthum
1
6
Hal. halimifolium halimifolium
7
Hal. lasiocalycinum rhiphaeum
Fossil constraint:
maxage = 11 Ma
Fossil constraint:
maxage = 5.3 Ma
Cistus crispus
Cistus heterophyllus
3
9
11
Cistus albidus
12
Cistus creticus
10
Cistus osbeckiifolius
Cistus horrens
13
Cistus ochreatus
8
Cistus symphytifolius
14
Cistus chinamadensis gomerae
Hal. umbellatum umbellatum
Cistus clusii clusii
15
17
Cistus clusii multiflorus
18
Cistus munbyi
16
Cistus libanotis
Cistus salviifolius
20
Cistus ladanifer africanus
19
21
Cistus ladanifer ladanifer
22
Cistus ladanifer sulcatus
23
Cistus monspeliensis
Cistus pouzolziii
25
Cistus populifolius major
24
26
Cistus populifolius populifolius
Cistus parviflorus
28
Cistus albanicus
27
Cistus psilosepalus
29
Cistus laurifolius
11
4
2
0 Ma
Miocene
Pliocene
Pleistocene

species in *C. clusii* lineage, in contrast to the 10 species (13 taxa) generated in the *C. libanotis* group (Fig. 4). Alternatively, rapid radiation is also interpreted as high rates of differentiation in comparison to those of flowering plants. The estimated rate of diversification in the *C. libanotis* lineage was significantly high (2.15-3.51 species per million years) compared to the median rate of diversification of angiosperm families (0.12 species per million years; with a maximum of 0.39) (Eriksson & Bremer 1992) and to that found in the Andean Valeraniaceae (0.80-1.34 species/Myr; Bell & Donoghue 2005), but similar to the explosive radiation of Andean *Lupinus* (1.93 - 2.78 species/Myr; Hughes & Eastwood 2006). As predicted, rapid diversification in the *C. libanotis* lineage is closely related with a combination of different sources of evidence prior to performing explicit analysis of radiation: (1) lack of resolution and low support values depicted mainly in the parsimony-based tree (Fig. 1A), which are overcome by increasing the number of DNA sequence fragments; (2) low resolution at the core of the haplotype network (Fig. 3); (3) short branch lengths and low pairwise sequence divergence (Fig. 4, Table 2).

In addition to evidence for common ancestry and rapid diversification, the fit of the diverse phenotypes observed in a lineage with their environment is necessary in prediction of adaptive radiations (Schluter 2000). Our character reconstruction suggests that shifts in leaf features allowing the colonization of different habitats have been related with specific speciation events (Fig. 2). Acquisition of diverse leaf features is associated with recent lineage splits, and thus closely related taxa exhibit different leaf morphologies (Givnish *et al.* 1995). Our character state optimization reveals that the

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←

Fig. 4. Phylogenetic chronogram of the *Cistus-Halimium* complex based on the Bayesian consensus tree. Fossil calibration points are indicated on the tree. Shaded area (green) delineates the establishment of the Mediterranean climate (Suc *et al.* 1995). Geological timescales are shown both at the top and the bottom. Photographs illustrate diversity in leaf morphology of the white-flowered *Cistus* species (as infraspecific taxa considered only one leaf morphology has been illustrated; i. e. *C. ladanifer* subsp. *ladanifer*, *C. clusii* subsp. *clusii*, *C. populifolius* subsp. *populifolius*). Species insolation conditions (Dansereau 1958) are plotted on the right side of the tree (○, helioxerophyllous and subhelioxerophyllous; ☁, subsciophyllous and submesophyllous).

most common recent ancestors of four sister species diversified in different environmental conditions (Fig. 2D, 2E, 2F) by means of shifts in leaf shape (Fig. 2A), leaf pubescence (Fig. 2C) and, in at least in two of them, leaf labdanum secretion (Fig. 2B). In fact, trends of correlated evolution (Table 5) between leaf traits and at least one ecological trait (habit, insolation conditions) have been found. The barely correlated evolution found suggests that shifts in environmental conditions must parallel evolutionary changes in *Cistus* leaf morphology as a whole and not in individual leaf features. Further studies testing correlated evolution of all leaf traits should be performed to analyse compensatory effects (trade-off). Alternatively, another strong indication of the adaptive value of a trait is when phylogenetically separate, but ecologically similar, species converge or show parallel patterns of variation along similar ecological gradients (Endler 1986). Many of the leaf morphological character-states that have been studied across the lineage (shape, labdanum secretion, pubescence) have been independently acquired at least twice (Fig. 2). An adaptation to different insolation, drained and competition conditions, mainly in the form of differences in leaf features, appears to have occurred within the white-flowered *Cistus*. In fact, helioxerophyllous species (*C. clusii*, *C. munbyi*) show ancestral, linear leaves while species with broad, thin leaves inhabit shadier environments (*C. laurifolius*, *C. populifolius*) (Table 3, Fig. 2).

Evidence that some morphological and/or physiological traits of species are particularly useful is the fourth necessary condition to describe a radiation as adaptive (trait utility, Schluter 2000). The adaptive implications of leaf size and shape differences are well documented (Givnish 1979; Givnish *et al.* 2004). Although our six DNA sequence data set render certain phylogenetic uncertainty for some sister species relationships because of moderate support (Fig. 1), the most plausible hypothesis agrees also with low character-state reconstruction uncertainty of leaf morphological utility using MacClade optimization and BayesTraits analysis of trait evolution. Leaf size and shape are implicated in important aspects as thermoregulation (Gates *et al.* 1968; Szwarcbaum 1982), efficiency of water use (Parkhurst & Loucks 1972; Cunningham *et al.*

1999), photosynthetic potential (Cunningham & Strain 1969), branching and rooting strategies (Givnish & Vermeji 1976), among others. Moreover, comparative studies have revealed the existence of well-marked ecological trends (Givnish 1987). Small leaf size (specifically, narrow leaves) are generally favoured under high exposure and/or low water availability as they help to maintain favourable leaf temperature and improves water use efficiency (Parkhurst & Loucks 1972; Givnish & Vermeji 1976). In fact, in the chaparral community (Ackerly *et al.* 2002) small-leaved species are concentrated at the high exposure end on south-facing slopes (*Adenostoma fasciculatum, Ceanothus cuneatus, Lotus scoparius, Artemisia californica*). Although our character reconstruction hypothesis indicates dynamic shifts of leaf shapes, the ancestral state (narrow leaves) appear to have evolved early into linear-lanceolate to elliptic, and then into ovate (plus ovate-lanceolate) leaves independently four times. Leaf shape is not however the only phenotypic trait associated with adaptation to dry conditions. Leaf pubescence is reported to be an adaptation to sunnier and hotter environments by reducing transpiration, increasing the probability of water uptake by leaves, maintaining favourable leaf temperature, and protecting against UV-B radiation responsible for photosynthetic inhibition (Ehleringer & Clark 1988; Savé *et al.* 2000). Accordingly, a combination of leaf trait strategies can meet in unrelated species. For instance, the ovate leaves of *C. parviflorus* unsuitable for xeric environments are protected by a dense tomentum of stellate hairs. In addition, leaves can be highly reflective in the visible spectrum by covering the upper surface with labdanum, and then decreasing transpiration. The high leaf secretion of resins (labdanum) in the linear-lanceolate leaves of *C. monspeliensis* and *C. ladanifer* may confer a trade-off compared to the narrower leaves of *C. clusii, C. mumbyi* and *C. libanotis* displaying lower labdanum concentration (Fig. 2). Further studies are needed to pinpoint whether combination of multiple leaf strategies are equally fit in dry, Mediterranean habitats suffering from dry hot summers and high solar radiation.

In summary, the evolutionary history of the 16 taxa (12 species) of the white-flowered *Cistus* in the last million year fits into utilization of the niche space in a novel

manner far after the Mediterranean climate establishment (2.8 Ma; see Suc *et al.* 1995). Multiple leaf strategies were essayed in the course of speciation not only to succeed in particular environments, but also to become some of the 10 species part of the dominant element in the Mediterranean scrub. A Mediterranean *Cistus* ancestor with linear, medium labdanum content and glabrous or subglabrous leaves may have spawn new lines of evolution exploiting seven pre-existing Mediterranean habitats. As far as we know, this is the first documented plant group involved in adaptive radiation process in the Mediterranean region.

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References

Ackerly DD, Knight CA, Weiss SB, Barton K, Starmer KP (2002) Leaf size, specific leaf area and microhabitat distribution of chaparral woody plants: contrasting patterns in species level and community level analyses. *Oecologia* **130**, 449-457.

Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap? a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**, 255-266.

Arrington JM, Kubitzki K (2003) Cistaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 62-70. Springer, Berlin.

Baldwin BG, Robichaux RH (1995) Historical biogeography and ecology of the Hawaiian silversword alliance (Asteraceae): new molecular phylogenetic perspectives. In: *Hawaiian biogeography: evolution on a hotspot archipelago* (eds. Wagner WL, Funk VA), pp. 257-287. Smithsonian Institution Press, Washington, D.C.

Bell CD, Donoghue MJ (2005) Phylogeny and biogeography of Valerianaceae (Dipsacales) with special reference to the South American valerians. *Organisms Diversity and Evolution* **5**, 147-159.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Cunningham GL, Strain BR (1969) An ecological significance of seasonal leaf variability in a desert shrub. *Ecology* **50**, 400-408.

Cunningham SA, Summerhayes BA, Westoby M (1999) Evolutionary divergences in leaf structure and chemistry, comparing rainfall and soil nutrient gradients. *Ecological Monographs* **69**, 569-588.

Daget P (1977) Le bioclimat méditerranéen caractères generaux, mode de caracterisation. *Vegetatio* **31**, 1-20.

Dansereau P (1958) Notes sur les Cistes. III: Les conditions de la distribution géographique. *Comptes rendus Séances Société Biogéographie* **304**, 22-25.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Ehleringer JR, Clark C (1988) Evolution and adaptation in *Encelia* (Asteraceae). In: *Plant evolutionary biology* (eds. Gottlieb LD, Subodh KJ), pp. 221-248. Chapman and Hall Ltd, New York.

Emshwiller E, Doyle JJ (1999) Chloroplast-expressed Glutamine synthetase (ncpGS): Potencial utility for phylogenetic studies with an example from *Oxalis* (Oxalidaceae). *Molecular Phylogenetics and Evolution* **12**, 310-319.

Endler JA (1986) *Natural selection in the wild*. Princeton University Press, Princeton, New Jersey.

Eriksson O, Bremer B (1992) Pollination systems, dispersal modes, life forms, and diversification rates in angiosmperm families. *Evolution* **46**, 258-266.

Gamage DT, de Silva MP, Inomata N, Yamazaki T, Szmidt AE (2006) Comprehensive molecular phylogeny of the sub-family Dipterocarpoideae (Dipterocarpaceae) based on chloroplast DNA sequences. *Genes and genetic system* **81**, 1-2.

Gamage DT, de Silva MP, Yoshida A, Szmidt AE, Yamazaki T (2003) Molecular phylogeny of Sri Lankan Dipterocarpaceae in relation to other Asian Dipterocarpaceae based on chloroplast DNA sequences. *Tropics* **13**, 79-87.

Gates DM, Alderfer R, Taylor SE (1968) Leaf temperature of desert plants. *Science* **159**, 994-995.

Givnish TJ (1979) On the adaptive significance of leaf form. In: *Topics in plant population biology* (eds. Solbrig OT, *et al.*), pp. 375-407. Columbia University Press, New York.

Givnish TJ (1984) Leaf and canopy adaptations in tropical forests. In: *Physiological ecology of plants of the wet tropics* (eds. Medina E, *et al.*), pp. 51-84. Junk, The Hague.

Givnish TJ (1987) Comparative studies of leaf form: assessing the relative roles of selective pressures and phylogenetic constraints. *New Phytologist* **106 (Suppl.)**, 131-160.

Givnish TJ, Evans TM, Zjhra ML, *et al.* (2000) Molecular evolution, adaptive radiation, and geographic diversification in the amphiatlantic family Rapateaceae: evidence from *ndh*F sequences and morphology. *Evolution* **54**, 1915-1937.

Givnish TJ, Knox E, Patterson TB, *et al.* (1996) The Hawaiian lobelioids are monophyletic and underwent a rapid initial radiation roughly 15 million years ago. *American Journal of Botany (suppl.)* **83**, 159.

Givnish TJ, Montgomery RA, Goldstein G (2004) Adaptive radiation of photosynthetic physiology in the Hawaiian Lobeliads: light regimes, static light responses, and whole-plant compensation points. *American Journal of Botany* **91**, 228-246.

Givnish TJ, Sytsma KJ, Smith JF, Hahn WJ (1995) Molecular evolution, adaptive radiation, and geographic speciation in *Cyanea* (Campanulaceae, Lebelioideae). In: *Hawaiian biogeography* (eds. Wagner WL, Funk VA), pp. 288-337. Smithsonian Institution Press, Washington.

Givnish TJ, Vermeji GJ (1976) Sizes and shapes of liane leaves. *The American naturalist* **110**, 743-778.

Greuter W (1996) Proposal to conserve the name *Cistus albanicus* (Cistaceae). *Taxon* **45**, 715-716.

Grosser W (1903) Cistaceae. In: *Dans Pflanzenreich* (ed. Engler A), p. 161, Berlin.

Gülz PG, Herrmann T, Hangst K (1996) Leaf trichomes in the genus *Cistus*. *Flora* **191**, 85-104.

Guzmán B, Vargas P (2005) Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trn*L-*trn*F, and *mat*K sequences. *Molecular Phylogenetics and Evolution* **37**, 644-660.

Hamilton M (1999) Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**, 521-523.

Hodges SA (1997) Rapid radiation due to a key innovation in columbines (Ranunculaceae: *Aquilegia*). In: *Molecular evolution and adaptive radiation* (eds. Givnish TJ, Sytsma KJ), pp. 391-405. Cambridge University Press, New York, USA.

Hughes C, Eastwood R (2006) Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences* **103**, 10334-10339.

Hughes CE, Bailey CD, Harris SA (2002) Divergent and reticulate species relationships in Leucaena (Fabaceae) inferred from multiple data sources: insights into polyploid origins and nrDNA polymorphism. *Am. J. Bot.* **89**, 1057-1073.

Johnson LA, Soltis DE (1994) *matK* DNA and phylogenetic reconstruction in Saxifragaceae s. str. *Systematic Botany* **19**, 143-156.

Kass R, Raftery A (1995) Bayes factors. *Journal of the American Statistical Association* **90**.

Kendall DG (1949) Stochastic processes and population growth. *Journal of the Royal Statistical Society B* **11**, 230-264.

Kim S-C, Crawford DJ, Francisco-Ortega J, Santos-Guerra A (1996) A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: evidence for extensive radiation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7743-7748.

Lowrey TK (1995) Phylogeny, adaptive radiation, and biogeography of Hawaiian *Tetramolopium* (Asteraceae, Astereae). In: *Hawaiian biogeography* (eds. Wagner WL, Funk VA), pp. 195-220. Smithsonian Institution Press, Washington.

Maddison WP, Maddison DR (1992) *MacClade: Analysis of Phylogeny and Character Evolution, versión 3.01*. Sinauer, Sunderland, MA.

Magallón SA, Sanderson MJ (2005) Angiosperm divergence times: The effect of genes, codon positions, and time constraints. *Evolution* **59**, 1653-1670.

Martín Bolaños M, Guinea E (1949) *Jarales y Jaras (Cistografia Hispanica)*. Ministerio de Agricultura. Direccion General de Montes, Caza y Pesca Fluvial. Instituto Forestal de Investigaciones y Experiencias, Madrid.

Meimberg H, Abele T, Brauchler C, *et al.* (2006) Molecular evidence for adaptive radiation of *Micromeria* Benth. (Lamiaceae) on the Canary Islands as inferred from chloroplast and nuclear DNA sequences and ISSR fingerprint data. *Molecular Phylogenetics and Evolution* **41**, 566-578.

Menke B (1976) Pliozäne und ältestquartäre Sporen- und pollenflora von Schleswig-Holstein. *Geologisches Jahrbuch. Reihe A.* **32**, 3-197.

Mooney HA, Dunn EL (1970) Photosynthetic systems of Mediterranean-climate shrubs and trees of California and Chile. *The American naturalist* **104**, 447-453.

Moran PA (2000) Estimation methods for evolutive processes. *Journal of the Royal Statistical Society B* **13**, 141-146.

Mort ME, Soltis PS, Soltis DE, Mabry M (2000) Comparison of three methods for estimating internal support on phylogenetic trees. *Systematics Biology* **49**, 160-171.

Nahal I (1981) The mediterranean climate from a biological viewpoint. In: *Ecosystems of the World 11, Mediterranean-type shrublands* (eds. de Castri F, *et al.*), pp. 63-86. Elsevier Scientific Publising Company, Amsterdam.

Naud G, Suc JP (1975) Contribution à l'étude paléofloristique des Coirons (Ardèche). Première analyses polliniques dans les alluvions sous-basaltique et interbasaltiques de Mirabel (Miocène supérieur). *Bulletin de la Société Géologique de France* **17**, 820-827.

Nylander JAA (2002) MrModeltest v1.0b. Department of Systematic Zoology. Uppsala University, Uppsala.

Pagel M, Meade A (2007) BayesTraits. Version 1.0.

Parkhurst D, Loucks O (1972) Optimal leaf size in relation to environment. *Journal of Ecology* **60**, 505-537.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Read J, Sanson GD (2003) Characterizing sclerophylly: the mechanical properties of a diverse range of leaf types. *New Phytologist* **160**, 81-99.

Reinthal PN, Meyer A (1997) Molecular phylogenetic tests of speciation models in lake Malawi cichlid fishes. In: *Molecular evolution and adaptive radiation* (eds. Givnish TJ, Sytsma KJ), pp. 375-390. Cambridge University Press, Melbourne, Australia.

Robichaux RH, Carr GD, Liebman M, Pearcy RW (1990) Adaptive radiation of the Hawaiian silversword alliance (Compositae-Madiinae): ecological, morphological, and physiological diversity. *Annals of the Missouri Botanical Garden* **77**, 64-72.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Rüber L, Van Tassell JL, Zardoya R (2003) Rapid speciation and ecological divergence in the American seven-spined Gobies (Gobiidae, Gobiosomatini) inferred from a molecular phylogeny. *Evolution* **57**, 1584-1598.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution* **19**, 101-109.

Savé R, Biel C, de Herralde F (2000) Leaf pubescence, water relations and chlrorophyll fluorescence in two species of Lotus creticus L. *Biologia Plantarum* **43**, 239-244.

Savolainen V, Chase MW, Hoot SB, *et al.* (2000) Phylogenetics of flowering plants based on combined analysis of plastid *atpB* and *rbc*L gene sequences. *Systematic Biology* **49**, 306-362.

Schimper AFW (1903) *Plant geography upon a physiological basis.* Clarendon Press, Oxford, UK.

Schluter D (2000) *The ecology of adaptive radiation.* Oxford University Press Inc., New York.

Skelton PW (1993) Adaptive radiation: definition and diagnostic tests. In: *Evolutionary patterns and processes* (eds. Lees DR, Edwards D), pp. 45-58. Academic Press, London.

Suc JP, Bertini A, Combourieu-Nebout N, *et al.* (1995) Structure of West Mediterranean vegetation and climate since 5.3 Ma. *Acta Zoologica Cracoviensia* **38**, 3-16.

Sun Y, Skinner DZ, Liang GH, Hulbert SH (1994) Phylogenetic analysis of *Sorghum* and related taxa using Internal Transcribed Spacer of nuclear ribosomal DNA. **89**, 26-32.

Swofford DL (1999) *"PAUP: phylogenetic analysis using parsimony," Version 4.0.* Illinois Natural History Survey, Champaign, Illinois.

Szwarcbaum I (1982) Influence of leaf morphology and optical properties on leaf temperature and survival in three Mediterranean shrubs. *Plant Science Letters* **26**, 47-56.

Taberlet P, Gielly L, G. P, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105-1109.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619-633.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876-4882.

Warburg EF (1968) *Cistus* L. In: *Flora Europaea* (eds. Tutin TG, *et al.*), pp. 282-285. Cambridge University Press, Cambridge.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (eds. Innis M, *et al.*), pp. 315-322. Academic Press, San Diego.

Appendix 1

Table of GenBank accession numbers

Appendix 1. GenBank accession numbers

Taxon	*trnL-trnF* accession no.	*trnK-matK* accession no.	*trnS-trnG* accession no.	*rbcL* accession no.	*ncpGS* accession no.	*ITS* accession no.
Cistus L.						
Cistus albanicus E.F. Warb. ex Heywood	DQ093057	DQ093010	Forthcoming	Forthcoming	Forthcoming	DQ092964
Cistus albidus L	DQ093021	DQ092974	Forthcoming	Forthcoming	Forthcoming	DQ092932
Cistus chinamadensis Bañares et Romero	DQ093033	DQ092986	Forthcoming	Forthcoming	Forthcoming	DQ092942
Cistus clusii Dunal subsp. *clusii*	DQ093056	DQ093009	Forthcoming	Forthcoming	Forthcoming	DQ092963
Cistus clusii Dunal subsp. *multiflorus* Demoly	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-	-
Cistus creticus L.	DQ093025	DQ092978	Forthcoming	Forthcoming	Forthcoming	DQ092936
Cistus crispus L.	DQ093060	DQ093013	Forthcoming	Forthcoming	Forthcoming	DQ093060
Cistus heterophyllus Desf.	DQ093036	DQ092989	Forthcoming	Forthcoming	Forthcoming	-
Cistus horrens Demoly	Forthcoming	Forthcoming	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Cistus ladanifer L. subsp. *africanus*	DQ093048	DQ093001	Forthcoming	Forthcoming	Forthcoming	DQ092956
Cistus ladanifer L. subsp. *ladanifer*	DQ093043	DQ092996	Forthcoming	Forthcoming	Forthcoming	DQ092951
Cistus ladanifer L. subsp. *sulcatus*	DQ093046	DQ092999	Forthcoming	Forthcoming	Forthcoming	DQ092954
Cistus laurifolius L.	DQ093052	DQ093005	Forthcoming	Forthcoming	Forthcoming	DQ092959
Cistus libanotis L.	DQ093040	DQ092993	Forthcoming	Forthcoming	Forthcoming	DQ092948
Cistus monspeliensis L.	DQ093059	DQ093012	Forthcoming	Forthcoming	Forthcoming	DQ092966
Cistus munbyi Pomel	DQ093053	DQ093006	Forthcoming	Forthcoming	Forthcoming	DQ092960
Cistus ochreatus C. Sm. ex Buch	DQ093032	DQ092985	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Cistus osbeckiifolius Webb ex Christ	Forthcoming	Forthcoming	Forthcoming	Forthcoming	Forthcoming	Forthcoming
Cistus parviflorus Lam.	DQ093023	DQ092976	Forthcoming	Forthcoming	Forthcoming	DQ092934
Cistus populifolius L. subsp. *major* (Dunal) Heywood	DQ093049	DQ093002	Forthcoming	Forthcoming	Forthcoming	DQ092957
Cistus populifolius L. subsp. *populifolius*	DQ093050	DQ093003	Forthcoming	Forthcoming	Forthcoming	-
Cistus pouzolzii Delile	DQ093054	DQ093007	Forthcoming	Forthcoming	Forthcoming	DQ092961
Cistus psilosepalus Sweet	DQ093041	DQ092994	Forthcoming	Forthcoming	Forthcoming	DQ092949
Cistus salviifolius L.	DQ093037	DQ092990	Forthcoming	Forthcoming	Forthcoming	DQ092945
Cistus symphytifolius Lam.	DQ093030	DQ092983	Forthcoming	Forthcoming	Forthcoming	DQ092940
Fumana (Dunal) Spach						
Fumana thymifolia (L.) Spach ex Webb	DQ093015	DQ092968	Forthcoming	Forthcoming	-	DQ092926
Halimium (Dunal) Spach						
Halimium atlanticum Humbert & Maire	Forthcoming	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-
Halimium atriplicifolium (Lam.) Spach	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-	-
Halimium calycinum (L.) K. Koch	DQ093020	DQ092973	Forthcoming	Forthcoming	Forthcoming	DQ092931
Halimium halimifolium (L.) Willk. *halimifolium*	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-	-
Halimium lasiocalicynum (Boiss. & Reut.) Gross ex Engl. subsp. *riphaeum* (Pau & Font Quer) Maire	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-	Forthcoming
Halmium ocymoides (Lam.) Willk.	Forthcoming	Forthcoming	Forthcoming	Forthcoming	-	-
Halimium umbellatum (L.) Spach	DQ093014	DQ092972	Forthcoming	Forthcoming	-	DQ092930
Helianthemum Mill.						
Helianthemum squamatum (L.) Dum. Cours.	DQ093016	DQ092969	Forthcoming	Forthcoming	Forthcoming	DQ092927
Tuberaria Dunal						
Tuberaria guttata (L.) Fourr.	DQ093018	DQ092971	Forthcoming	Forthcoming	-	DQ092929

Appendix 2

Table of results of Bayesian inference of trait evolution

Appendix 2. Bayesian inference of trait evolution of successive iterations of the chain (9,000,000) in the white-flowered *Cistus* lineage by reversible jump Markov chain Monte Carlo. Means ± confidence intervals (95%) of the log-likelihoods (Lh) and rate coefficients are shown

Trait	Ratedev	Log-Likelihood (Lh)	qAB	qAC	qAD	qAE	qAF	qAG	qBA	qBC	qBD	qBE	qBF	qBG	qCA	qCB	qCD	qCE
Leaf shape	90	-14.62 ± 0.00	39.04 ± 0.19	31.08 ± 0.20	34.06 ± 0.20	-	-	-	35.66 ± 0.19	49.08 ± 0.16	44.54 ± 0.17	-	-	-	38.19 ± 0.20	40.96 ± 0.21	37.90 ± 0.20	-
Labdanum secretion	120	-11.46 ± 0.01	39.34 ± 0.23	44.69 ± 0.23	-	-	-	-	45.74 ± 0.23	48.30 ± 0.23	-	-	-	-	48.91 ± 0.23	46.43 ± 0.23	-	-
Leaf pubescence	130	-13.18 ± 0.01	60.89 ± 0.20	62.37 ± 0.19	-	-	-	-	42.76 ± 0.24	37.79 ± 0.24	-	-	-	-	44.09 ± 0.25	44.18 ± 0.25	-	-
Soil	110	-8.45 ± 0.00	33.70 ± 0.14	26.83 ± 0.15	-	-	-	-	24.22 ± 0.17	28.10 ± 0.17	-	-	-	-	24.12 ± 0.17	32.26 ± 0.17	-	-
Insolation conditions	100	-12.55 ± 0.01	35.55 ± 0.19	33.81 ± 0.19	33.96 ± 0.19	-	-	-	31.43 ± 0.19	34.59 ± 0.19	43.55 ± 0.19	-	-	-	34.20 ± 0.20	36.69 ± 0.20	35.38 ± 0.20	-
Environment	100	-19.03 ± 0.06	45.13 ± 0.23	45.89 ± 0.23	44.40 ± 0.23	44.92 ± 0.23	44.60 ± 0.22	44.43 ± 0.22	44.22 ± 0.23	47.19 ± 0.22	44.89 ± 0.23	46.88 ± 0.22	44.36 ± 0.23	46.13 ± 0.22	41.50 ± 0.22	46.10 ± 0.21	42.84 ± 0.22	45.21 ± 0.22

Trait	qCF	qCG	qDA	qDB	qDC	qDE	qDF	qDG	qEA	qEB	qEC	qED	qEF	qEG	qFA	qFB	qFC	qFD
Leaf shape	-	-	33.96 ± 0.20	37.56 ± 0.20	33.52 ± 0.20	-	-	-	-	-	-	-	-	-	-	-	-	-
Labdanum secretion	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leaf pubescence	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Soil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Insolation conditions	-	-	21.86 ± 0.17	39.73 ± 0.19	24.31 ± 0.17	-	-	-	-	-	-	-	-	-	-	-	-	-
Environment	42.33 ± 0.22	43.14 ± 0.22	46.39 ± 0.23	48.07 ± 0.23	48.92 ± 0.22	48.04 ± 0.23	47.30 ± 0.22	46.50 ± 0.22	44.92 ± 0.23	47.82 ± 0.22	47.81 ± 0.22	45.61 ± 0.22	45.63 ± 0.22	45.63 ± 0.23	46.36 ± 0.23	47.64 ± 0.23	48.23 ± 0.23	46.79 ± 0.22

Trait	qFE	qFG	qGA	qGB	qGC	qGD	qGE	qGF
Leaf shape	-	-	-	-	-	-	-	-
Labdanum secretion	-	-	-	-	-	-	-	-
Leaf pubescence	-	-	-	-	-	-	-	-
Soil	-	-	-	-	-	-	-	-
Insolation conditions	-	-	-	-	-	-	-	-
Environment	47.31 ± 0.23	46.18 ± 0.23	46.39 ± 0.23	49.50 ± 0.22	48.75 ± 0.23	46.66 ± 0.22	47.99 ± 0.22	46.76 ± 0.22

Appendix 3

Table of haplotypes found in 12 species and subspecies of the white-flowered *Cistus* lineage

Appendix 3. List of haplotypes found in 12 species and subspecies of the white-flowered *Cistus* lineage. Variable sites of the sequences of four plastid DNA fragments (*trnL-trnF, rbcL, trnK-matK, trnS-trnG*) are shown. Nucleotide position for each data set is numbered from the 5′ to the 3′ DNA ends

	trnL-trnF																					
Nucleotide position	**7**	**8**	**29**	**82**	**116**	**148**	**163**	**177**	**223**	**263**	**270**	**288**	**323**	**359**	**363**	**365**	**366**	**367**	**368**	**380**	**413**	**422**
Haplotype																						
C. albanicus	C	A	T	G	A	A	C	G	A	C	A	T	-	T	-	-	-	-	-	-	G	G
C. ladanifer africanus	C	A	T	G	A	G	C	G	A	C	T	T	-	T	-	-	-	-	-	-	G	G
C. ladanifer ladanifer & sulcatus	C	A	T	G	A	G	C	G	A	C	T	T	-	T	-	-	-	-	-	-	G	G
C. laurifolius	C	A	T	G	A	G	C	C	A	C	A	T	A	A	A	A	T	T	T	T	T	G
C. libanotis	C	A	T	G	A	G	C	G	A	C	A	T	-	-	-	-	-	-	-	T	G	G
C. monspeliensis	C	A	T	G	G	G	T	C	C	A	A	T	T	T	T	T	T	A	A	G	G	C
C. parviflorus	T	A	T	A	A	G	C	C	A	C	A	G	T	A	A	A	T	T	T	A	T	G
C. populifolius major	C	A	T	G	A	G	C	C	A	C	A	T	T	T	A	A	A	T	T	T	G	C
C. populifolius populifolius	C	A	T	G	A	G	C	C	A	C	A	T	T	T	A	A	A	T	T	T	G	C
C. pouzolzii	C	C	T	G	A	G	C	C	A	C	A	T	T	T	A	A	A	T	T	-	G	G
C. psilosepalus	C	A	T	G	A	G	C	C	C	C	A	T	T	A	A	A	T	T	T	T	T	G
C. salviifolius	T	A	C	G	A	G	C	G	A	C	T	T	-	T	-	-	-	-	-	-	G	G

	rbcL												
Nucleotide position	**174**	**376**	**378**	**621**	**717**	**795**	**916**	**934**	**1046**	**1069**	**1297**	**1305**	**1320**
Haplotype													
C. albanicus	C	A	C	C	G	T	C	G	T	C	C	C	G
C. ladanifer africanus	T	G	C	T	G	C	C	A	A	C	C	A	G
C. ladanifer ladanifer & sulcatus	T	G	C	T	G	C	C	A	A	C	C	A	G
C. laurifolius	C	A	C	C	A	T	C	G	T	C	C	C	G
C. libanotis	T	A	C	T	G	C	C	A	A	C	C	C	G
C. monspeliensis	C	A	C	T	G	T	C	G	T	C	C	C	G
C. parviflorus	C	A	T	C	G	T	T	G	T	C	C	C	A
C. populifolius major	C	A	C	?	G	T	C	G	T	C	C	C	G
C. populifolius populifolius	C	A	C	T	G	T	C	G	T	C	C	C	G
C. pouzolzii	C	A	C	T	G	T	C	G	T	C	C	C	G
C. psilosepalus	C	A	C	C	G	T	C	G	T	T	C	C	G
C. salviifolius	T	A	C	T	G	C	C	A	A	C	A	C	G

Appendix 3 *(Continued)*

	trnK-matK														
Nucleotide position	**33**	**105**	**109**	**113**	**131**	**297**	**302**	**317**	**344**	**402**	**428**	**434**	**458**	**595**	**607**
Haplotype															
C. albanicus	G	C	G	A	T	G	T	T	C	C	G	G	G	G	G
C. ladanifer africanus	G	C	G	A	T	G	A	T	C	C	G	C	G	G	G
C. ladanifer ladanifer & sulcatus	G	C	G	A	T	G	A	T	C	C	G	C	G	G	G
C. laurifolius	G	A	G	A	C	G	T	T	T	C	T	G	T	G	T
C. libanotis	G	C	T	A	T	G	T	T	C	C	G	G	G	G	G
C. monspeliensis	G	A	G	A	T	G	T	T	C	C	G	G	G	G	G
C. parviflorus	G	C	T	A	T	G	T	T	C	A	G	G	G	G	G
C. populifolius major	G	A	G	C	T	G	T	T	C	C	G	G	G	G	G
C. populifolius populifolius	G	A	G	C	T	G	T	T	C	C	G	G	G	G	G
C. pouzolzii	G	A	G	A	T	G	T	T	C	C	G	G	G	G	G
C. psilosepalus	T	A	G	A	T	G	T	T	C	C	G	G	G	G	G
C. salviifolius	G	C	G	A	T	T	T	G	C	C	G	G	G	T	G

	trnK-matK													
Nucleotide position	**853**	**869**	**871**	**889**	**918**	**947**	**1012**	**1031**	**1089**	**1126**	**1159**	**1316**	**1323**	**1334**
Haplotype														
C. albanicus	G	T	G	C	T	C	G	A	C	A	A	C	C	A
C. ladanifer africanus	G	G	G	C	T	C	G	A	C	A	A	C	C	C
C. ladanifer ladanifer & sulcatus	G	G	G	C	T	C	G	A	T	A	A	C	C	C
C. laurifolius	G	G	G	C	T	C	G	A	C	A	A	C	C	C
C. libanotis	G	G	G	C	T	C	G	A	C	A	A	C	C	C
C. monspeliensis	T	G	A	C	T	C	G	A	C	A	A	C	C	C
C. parviflorus	G	G	G	C	T	C	G	A	C	A	A	C	C	C
C. populifolius major	G	G	G	C	G	C	G	A	C	A	G	C	C	C
C. populifolius populifolius	G	G	G	C	G	C	G	A	C	A	G	C	C	C
C. pouzolzii	G	G	G	C	T	C	T	A	C	A	A	C	C	C
C. psilosepalus	G	G	G	C	T	T	G	A	C	A	A	A	G	C
C. salviifolius	G	G	G	A	T	C	G	C	C	C	A	C	C	C

Appendix 3 *(Continued)*

	trnS-trnG																							
Nucleotide position	**102**	**104**	**105**	**118**	**126**	**155**	**158**	**217**	**227**	**236**	**255**	**303**	**312**	**333**	**342**	**350**	**366**	**367**	**414**	**423**	**436**	**528**	**533**	**569**
Haplotype																								
C. albanicus	T	G	C	T	T	C	T	C	T	T	C	A	T	C	T	T	G	A	C	A	G	T	T	G
C. ladanifer africanus	T	G	C	T	T	C	T	C	T	T	C	A	T	A	T	T	G	A	A	A	G	C	T	G
C. ladanifer ladanifer & sulcatus	T	G	C	T	T	C	T	C	T	T	C	A	T	A	T	G	G	A	A	C	G	C	T	G
C. laurifolius	T	G	C	T	G	C	T	C	T	T	C	A	T	A	T	T	G	A	C	A	G	C	T	G
C. libanotis	T	G	C	T	T	A	T	C	T	T	T	A	T	A	T	T	G	A	C	A	T	C	T	A
C. monspeliensis	T	G	C	C	T	C	T	C	T	T	C	A	G	A	T	T	T	T	C	A	G	C	A	G
C. parviflorus	T	G	C	T	T	C	T	C	T	T	C	A	G	C	T	T	G	A	C	A	G	T	T	G
C. populifolius major	G	G	C	T	T	C	G	C	T	T	C	T	T	A	T	T	G	A	C	A	G	C	T	G
C. populifolius populifolius	G	G	C	T	T	C	G	C	T	T	C	A	T	A	T	T	G	A	C	A	G	C	T	G
C. pouzolzii	T	G	C	T	T	C	T	C	T	T	C	A	T	A	T	T	T	A	C	A	G	C	T	?
C. psilosepalus	T	G	C	T	T	C	T	C	T	G	C	A	T	A	T	T	G	A	C	A	G	C	T	G
C. salviifolius	T	T	A	T	T	C	T	A	C	T	C	A	T	A	G	T	T	C	C	A	G	C	T	G

5

Unexpected synchronous differentiation in mainland and Canarian *Cistus*

Publicación: Guzman, B. & Vargas, P. Unexpected synchronous differentiation in mainland and Canarian *Cistus* (Enviado a Molecular Ecology).

Abstract

Diversification rates of insular oceanic lineages are considered to be higher than those of mainland counterparts. Five *Cistus* endemics to the Canary Islands have been reported to form a sister group to a clade of three mainland species. The facts that the number of species is somewhat higher on the oceanic islands and the two groups share a most recent common ancestor confer them an ideal study case to test early stages of accelerated evolution. Network analyses of plastid DNA haplotypes were performed using sequences of the *trnS-trnG* and the *trnK-matK* spacers from 70 individuals of the five Canarian and the three mainland species. Phylogenetic reconstructions and molecular clock estimates were additionally explored to infer haplotype relationships and ancestry and to estimate divergence times and differentiation rates. Net differentiation rates in the Canarian lineage parallel those of mainland *Cistus*, as revealed by similar diversity in species (5 vs. 3), haplotype (7 vs. 6) and haplotype-clade (4 vs. 4) numbers. Splitting dates in the Canarian (0.33 ± 0.14) and mainland (0.66 ± 0.18 Ma) lineages reveals a relatively similar tempo of net differentiation rates. All these results are interpreted as evidence of two synchronous evolutionary histories in the Canarian flora as *Cistus* ancestors coped with two hotspots of plant diversity: the Macaronesian and Mediterranean regions. Analysis of Canarian populations suggests that although moderate adaptive radiation (five species) cannot be ruled out from our data sets, a geographical rather than ecological isolation is argued to be primarily responsible for early stages of a non-adaptive radiation of the Canarian lineage.

Key words: Canary Islands, Cistaceae, *Cistus*, diversification rates, molecular clock, phylogeography, radiation

1. Introduction

The most common process described to explain the origin of species on oceanic islands is speciation associated with radiation. Traditionally, scholars accept as a working hypothesis that speciation rates are especially high in island groups due to formation of large numbers of species in short periods of time (Carlquist 1965, 1974; Sang *et al.* 1994; Kim *et al.* 1996; Crawford & Stuessy 1997; Francisco-Ortega *et al.* 1997; Baldwin *et al.* 1998; Grant 1998). Comparison of insular/continental sister lineages may ultimately allow for a more sensitive test for accelerated diversification (Baldwin *et al.* 1998). The considerable habitat diversity and the isolation of oceanic islands, compared to those of mainland, produce such a low competition and empty ecological niches that permit species proliferation (e.g., Hawaiian silversword alliance, Baldwin & Robichaux 1995; *Echium*, Böhle *et al.* 1996; Hawaiian lobelioids, Givnish *et al.* 1996; *Sonchus*, Kim *et al.* 1996; *Argyranthemum*, Francisco-Ortega *et al.* 1997). In addition, radiation is a rapid proliferation of species from a single ancestor that usually implies a great morphological and/or physiological divergence among species related to environment (adaptive radiation) or alternative (non-adaptive radiation) adaptations (Schluter 2003). Lamarck (1809), Darwin (1859), and Haeckel (1866) used the concept of adaptive radiation to link environment and organic evolution, although that term was formally proposed by Osborn (1902). Interdependence of environments, instead of morphology *per se*, and lines of evolution was explicitly proposed by Huxley (1942) in his definition of this concept. A new twist on a most specific definition of adaptive radiation was turned around by Simpson (1953) when a tempo scale was introduced. The most recent predictions of adaptive radiation include analysis of not only common ancestry, phenotype-environment and rapid speciation correlation, but also trait utility in the course of evolution (Schluter 2003).

Characteristics of *Cistus* offer an ideal opportunity to test levels of differentiation in mainland and the Canary Islands. The primarily Mediterranean genus *Cistus* contains 16 species with a distribution range extending from the Caucasus Mountains to northern Africa, although the centre of diversity is found in the western Mediterranean

region (Iberia and northern Africa) (Arrington & Kubitzki 2003). Additionally, five species are distributed across the Canary Islands (Table 1), which constitute one of the five volcanic archipelagos that form Macaronesia, together with Azores, Cape Verde, Madeira and Selvagems. Located on the northwestern Atlantic coast of Africa, the Canary archipelago, consists of seven islands (Lanzarote, Fuerteventura, Gran Canaria, Tenerife, La Gomera, La Palma, El Hierro) that exhibit a broad range of geological ages (from 1.12 Ma of El Hierro to 20.6 Ma of Fuerteventura) (Carracedo *et al.* 2002). The altitudinal gradients (from sea level to 3718 m) and the influence of the humid trade winds on the northern slopes of the seven islands have originated a high diversity of habitats responsible for the high degree of endemism (40%) found in the archipelago (Santos-Guerra 1999). These geographical and ecological conditions fostered ideal conditions to promote speciation in disparate plant groups of the Canarian flora (for revision see Carine 2005; Vargas 2007). Causes behind moderate levels of speciation in some genera as *Cistus* are however intriguing. *Cistus symphytifolius* has been recorded from pine and termophilous woodland of four islands (Tenerife, La Palma, La Gomera, El Hierro), whereas the other four species show a more limited ecology and distribution (*C. chinamadensis* in laurisilva of Tenerife and La Gomera; *C. horrens* and *C. ochreatus* in pine woodland of Gran Canaria; *C. osbeckiifolius* in high mountain habitats of Tenerife). Phylogenetic reconstructions of the genus *Cistus* revealed a single origin for the Canarian lineage and a close relationship with Mediterranean relatives. In particular, the Canarian lineage is sister to a mainland group of three purple-flowered species distributed in the Iberian Peninsula and northern Africa (*C. heterophyllus*) and the Mediterranean Basin (*C. albidus, C. creticus*).

Biogeographic studies that incorporate a temporal framework for diversification of island lineages and their mainland relatives provide competent hypotheses on the relationships between island and mainland floras. In this study we employed plastid haplotype variation of the five Canarian *Cistus* and their sister group of three mainland species to: (1) analyze phylogeographical relationships of populations and species; (2) infer patterns of historical differentiation within each group; (3) test whether

diversification of species and haplotype lineages was accelerated in the insular clade as compared to that of the mainland clade.

2. Material and methods

2.1. Sample strategy and DNA sequencing

A total of 70 samples representing 21 populations (43 individuals) of the five *Cistus* endemics to the Canary Islands and 18 populations (27 individuals) of three mainland purple-flowered species of *Cistus* were sampled to investigate plastid (*trnS-trnG, trnK-matK*) haplotype variation (Table 1). In addition, one sample of the two other purple/pinkish-flowered species (*C. crispus, C. parviflorus),* the ten white-flowered species, two *Halimium* species and one *Tuberaria* species were sequenced to investigate phylogenetic relationships among haplotypes and to estimate divergence times.

Standard primers were used for amplification and sequencing of the *trnK-matK* (*trnK*-3914F, *matK*-1470R) (Johnson & Soltis 1994) and the *trnS* (GCU)-*trnG* (UCC) (Hamilton 1999) spacers. One internal primer (*trnSG*polyTf) was designed to amplify and sequence the *trnS-trnG* spacer due to mononucleotide repeat stretches found in the purple-flowered species (poly-T at site 190, poly-A at site 580). The *trnSG*polyTf primer is a 20-nucleotide-long oligo (5'TTAGATTCTATTTACATTCT3'). Procedures used for DNA sequencing are given in Guzmán & Vargas (2005), except for specific amplifications. After 1-3 min pretreatment at 94 °C, PCR conditions for the *trnK-matK* and *trnS-trnG* amplifications were: 28 cycles of 1 min at 94 °C, 1-2 min at 50-53 °C and 1-2 min at 72 °C.

2.2. Haplotype data analysis

Sequences of *trnK-matK* and *trnS-trnG* were combined and aligned by hand given the low number of indels across sequences. Technical problems in the sequencing of *trnS-trnG* spacer caused by poly-T stretches forced us to eliminate 101 bp (190-291 bp sites). Relationships among Canarian haplotypes, in one hand, and mainland purple-flowered species, on the other, were inferred using the software TCS 1.21 (Clement *et al.* 2000).

Table 1. Cistaceae taxa sequenced for the *trnK-matK* and *trn*S-*trn*G DNA spacers. Material source, number of individuals per population[a], voucher reference, haplotype numbers and letters and GenBank accession numbers are also indicated. Taxonomy follows Guzmán & Vargas (2005)

Taxon	Locality/source (Number of individuals per population[a])	Voucher	Haplotype number /letter	*trnK-matK* accession no.	*trnS-trnG* accession no.
Cistus L.					
C. albanicus E.F. Warb. ex Heywood	Cultivated	R. G. Page 8cBGA04 (MA)	-	DQ093010	Forthcoming
Cistus albidus L.	Spain, Madrid, Aldea del Fresno	P. Vargas 25PV03 (MA)	A	DQ092974	Forthcoming
"	Spain, Cádiz, Grazalema	P. Vargas 252PV06 (MA)	A	Forthcoming	Forthcoming
"	Portugal, Sagres	B. Guzmán 34BGA04 (MA)	A	Forthcoming	Forthcoming
"	Morocco, Tetuán	P. Vargas 41PV03 (MA)	A	DQ092975	Forthcoming
"	Morocco, Ketama	B. Guzmán 111BGA04 (MA)	A	Forthcoming	Forthcoming
"	Morocco, Fardiwa (2)	J. Martínez 118JM03 (MA)	A	Forthcoming	Forthcoming
"	Morocco, Talamagait	B. Guzmán 94BGA04 (MA)	B	Forthcoming	Forthcoming
"	Morocco, Ouazanne (2)	J. Martínez 210JM04 (MA)	A, C	Forthcoming, Forthcoming	Forthcoming, Forthcoming
"	Morocco, Beni-Hadifa	B.Guzmán 105BGA04 (MA)	D	Forthcoming	Forthcoming
Cistus chinamadensis Bañares et Romero subsp. *gomerae*	La Gomera, Roque Agando	R. G. Page 144BGA04 (MA)	2	DQ092987	Forthcoming
"	La Gomera, Roque Agando (3)	A. Fernández & J. Leralta 44BGA04 (MA)	2	DQ09286	Forthcoming
Cistus chinamadensis Bañares et Romero subsp. *chinamadensis*	Tenerife, Chinamada (3)	C. Rodríguez 4BGA06 (MA)	1	Forthcoming	Forthcoming
Cistus clusii Dunal	Spain, Málaga, Mijas	R. G. Page 8bBGA04 (MA)	-	DQ093009	Forthcoming
Cistus creticus L.	Morocco, Azilal (5)	P. Vargas 68PV05 (MA)	A, C, F	Forthcoming, Forthcoming, Forthcoming	Forthcoming, Forthcoming, Forthcoming
"	Morocco, Afourer (3)	P. Vargas 7PV05 (MA)	C, E	Forthcoming, Forthcoming	Forthcoming, Forthcoming
"	Morocco, Asni	P. Vargas 86PV05 (MA)	E	Forthcoming	Forthcoming
"	Morocco, Essaouira (2)	P. Vargas 131PV03 (MA)	A, E	Forthcoming, Forthcoming	Forthcoming, Forthcoming
"	Greece, Olympus	P. Vargas 209PV04 (MA)	A	DQ092978	Forthcoming
"	Greece, Crete, Veneratos	P. Vargas 94PV05 (MA)	A	Forthcoming	Forthcoming
Cistus crispus L.	Spain, Córdoba, Posadas	B. Guzmán 58BGA04 (MA)	G	DQ093013	Forthcoming
Cistus heterophyllus L.	Morocco, Beni-Hadifa	B. Guzmán 99BGA04 (MA)	A	Forthcoming	Forthcoming
"	Morocco, Gurugú Mountain	B. Guzmán 84BGA04 (MA)	A	DQ092989	Forthcoming
"	Morocco, Targuist	B. Guzmán 108BGA04 (MA)	A	Forthcoming	Forthcoming
Cistus horrens Demoly	Gran Canaria, Ayacata (3)	B. Guzmán 2BGA05 (MA)	3, 4	Forthcoming, Forthcoming	Forthcoming, Forthcoming
"	Gran Canaria, San Bartolomé de Tirajana (3)	B. Guzmán 5BGA05 (MA)	3	Forthcoming	Forthcoming
Cistus ladanifer L.	Spain, Madrid, El Atazar	B. Guzmán 28BGA03 (MA)	-	Forthcoming	Forthcoming
Cistus laurifolius L.	Spain, Madrid, Las Rozas	P. Vargas 12PV03 (MA)	-	DQ093004	Forthcoming
Cistus libanotis L.	Spain, Córdoba	R. G. Page 149BGA04 (MA)	-	DQ092993	Forthcoming

Cistus monspeliensis L.	Portugal, Sagres	B. Guzmán 35BGA04(MA)	-	**Forthcoming**	**Forthcoming**
"	Tenerife, Villa de Arico	P. Vargas 42PV05 (MA)	-	**Forthcoming**	**Forthcoming**
Cistus munbyi Pomel	Morocco	O. Filippi 4BGA04 (MA)	-	DQ093006	**Forthcoming**
Cistus ochreatus C. Sm. ex Buch	Gran Canaria	R. G. Page 8BGA04 (MA)	1	DQ092985	**Forthcoming**
"	Gran Canaria	R. G. Page 150BGA04 (MA)	1	DQ092984	**Forthcoming**
"	Gran Canaria, Artenara (3)	P. Vargas 60PV05 (MA)	1	**Forthcoming**	**Forthcoming**
"	Gran Canaria, Tamadaba (3)	B. Guzmán 4BGA05	1	**Forthcoming**	**Forthcoming**
Cistus osbeckiifolius Webb ex Christ	Tenerife, Cañadas del Teide	P. Escobar 48/05 (MA)	5	**Forthcoming**	**Forthcoming**
"	Tenerife, Cañadas del Teide (3)	P. Vargas 177PV05 (MA)	5	**Forthcoming**	**Forthcoming**
Cistus parviflorus Lam.	Greece, Crete	O. Filippi 6BGA04 (MA)	-	DQ092976	**Forthcoming**
Cistus populifolius L.	Spain, Ávila, Arenas de San Pedro	P. Vargas 5PV03 (MA)	-	DQ093003	**Forthcoming**
Cistus pouzolzii Delile	Morocco, Ketama	S. L. Jury 698247MA	-	DQ093008	**Forthcoming**
Cistus psilosepalus Sweet	Spain, Ávila, Arenas de San Pedro	P. Vargas 7PV03 (MA)	-	DQ092994	**Forthcoming**
Cistus salviifolius L.	Spain, Ávila, Arenas de San Pedro	P. Vargas 6PV03 (MA)	-	DQ092990	**Forthcoming**
Cistus symphytifolius Lam	Tenerife, Vilaflor (2)	P. Vargas 174PV05 (MA)	2, 5	**Forthcoming, Forthcoming**	**Forthcoming, Forthcoming**
"	Tenerife, Villa de Arico	P. Vargas 41PV05 (MA)	5	**Forthcoming**	**Forthcoming**
"	Tenerife, Aguamansa	C. García 7CG05 (MA)	5	**Forthcoming**	**Forthcoming**
"	Tenerife, Barranco de las Ánimas	P. Escobar 51/05 (MA)	5	**Forthcoming**	**Forthcoming**
"	Tenerife, Güimar (2)	P. Vargas 48PV05 (MA)	7	**Forthcoming**	**Forthcoming**
"	Tenerife, Teide	P. Escobar 45/05 (MA)	7	**Forthcoming**	**Forthcoming**
"	Tenerife, Teno (5)	C. García 7CG06 (MA)	6, 7	**Forthcoming, Forthcoming**	**Forthcoming, Forthcoming**
"	La Palma, Barranco de Garome (2)	C. García 35CG05 (MA)	2	**Forthcoming**	**Forthcoming**
"	La Palma, La Cumbrecita	B. Guzmán 143BGA05 (MA)	2	DQ092983	**Forthcoming**
"	La Palma, Roque de los Muchachos (2)	V. Valcárcel 64VV05 (MA)	2	**Forthcoming**	**Forthcoming**
Halimium (Dunal) Spach					
Halimium ocymoides (Lam.) Willk.	Portugal, Coimbra	R. G. Page 158BGA04 (MA)	-	**Forthcoming**	**Forthcoming**
Halimium umbellatum (L.) Spach	Spain, Madrid, Tres Cantos	P. Vargas 71BGA04 (MA)	-	DQ092972	**Forthcoming**
Tuberaria Dunal					
Tuberaria guttata (L.) Fourr.	Portugal, Vila do Bispo	B. Guzmán 44BGA04 (MA)	-	DQ092971	**Forthcoming**

[a] One individual per population except when indicated in brackets.

The program implements a statistical parsimony approach using the algorithm described in Templeton et al. (1992) to construct haplotype networks. The maximum number of differences among haplotypes, as a result of single substitutions, was calculated with 95% confidence limits and treating gaps as missing data. Indel coding was cautiously considered as it has been documented a significant number of biased results (Provan *et al.* 2001).

2.3. Estimating lineages divergences

Tree topology and branch lengths from the combined data set of *trnS-trnG* and *trnK-matK* sequences were obtained using Bayesian Inference (BI) and Maximum Parsimony (MP). Bayesian Inference was analysed in Mr. Bayes 3.2.1 (Ronquist & Huelsenbeck 2003) using the simplest model of molecular evolution (GTR + G) selected by Mr.Modeltest (Posada & Crandall 1998; Nylander 2002). Two identical searches with three million generations each (chain temperature = 0.2; sample frequency = 100) were performed. In both runs probabilities converged on the same stable value approximately after generation 30,000. A 50% majority–rule consensus tree was calculated using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as estimate of robustness (Alfaro *et al.* 2003). Maximum Parsimony analyses were performed as in Guzmán & Vargas (2005), except for internal clade support. We performed a full heuristic bootstrap using 1000 replicates with random taxon addition, TBR branch swapping and the options Multrees and Steepest Descent in effect but limiting the number of rearrangements per random replicate to 10,000,000 due to computational limitations. To estimate divergence times we employed the 50% majority rule consensus tree obtained by Bayesian Inference.

To check the constancy of substitution rates we used the Langley and Fitch (LF) test (Magallón & Sanderson 2005). We rejected the null hypothesis of constant rate (χ^2 = 175.64; d.f. = 29) and, then, divergence times were estimated using the r8S 1.71 program (Sanderson 2002) with a Penalized Likelihood (PL) approach. Penalized Likelihood was implemented with the Truncated Newton (TN) algorithm. Initial results were obtained

under the following parameters: cvstart=0.5; cvinc=0.5; cvnum=10 with cross-validation enforced. The rate smoothing with the lowest crossvalidation scores was selected and the dating procedure was repeated with the following parameters: collapse; num_time_guesses= 5 and num_restarts=5. Crossvalidation suggested 32 as the best smooth parameter. Standard deviations were obtained by bootstrapping data using the Maximum Likelihood approach (PAUP*, Swofford 2002). Confidence intervals of estimated ages were calculated by bootstrapping the Bayesian consensus tree (Baldwin & Sanderson 1998; Sanderson & Doyle 2001). One thousand replicates of a full heuristic bootstrap (simple taxon addition sequence, Multrees, Steepest Descent, TBR branch swapping, holding one tree at each step) were obtained enforcing a topological constrained to obtain trees with the same topology (Bayesian consensus tree) but different branch lengths and using the GTR+G substitution model. To convert relative divergence times into absolute time units we used the split age between the complex *Cistus-Halimium* and the rest of the Cistaceae, obtained in a previous study (B. Guzmán & P. Vargas, unpublished; after Wikström *et al.* 2001), to constraint the node with a maximum age of at 3.26 Ma. A likelihood ratio test was performed in r8s (rrlike command) to test whether the Canarian and the mainland lineages have evolved from the most recent ancestor at the same constant rate.

Alternatively, divergence time estimates and associated confidence intervals (Kishino *et al.* 2001) were obtained utilizing a relaxed Bayesian molecular clock method, which allows for variability in the evolutionary rate over time. We used the Multidistribute package (http://statgen.ncsu.edu/thorne/multidivtime.html, contents italicized below) developed by Thorne et al. (1998) and Thorne and Kishino (2002). We followed the procedure outline in Rutschmann (2005) which implies three steps. First, the baseml program of the PAML package (version 3.15, Yang 1997) calculated the parameters of the substitution model (base frequencies, transition/transversion rate ratio and rate heterogeneity among sites) under the F84 model (Kishino & Hasegawa 1989). Second, the *paml2modelinf* was run to convert the baseml output to useable data for the *Estbranches* program (Thorne *et al.* 1998) which produced maximum likelihood

estimates of branch lengths within the constrained topology and their variance–covariance matrix. Third, the application *Multidivtime* (Thorne and Kishino 2002) was used to incorporate the results of *Estbranches* and the calibration to determine the posterior estimates of clade divergence times. *Multidivtime* parameters were set according to the recommendations of the program instructions. The mean of the prior distribution of time from the ingroup root (the most recent common ancestor (MRCA)) to present (rttm) and its standard deviation (rttmsd) was set to 0.32 (where 1.0 time unit = 10 Ma). This was based on the estimated age of the split between *Tuberaria* genus and the *Cistus-Halimium* assemblage estimated in a previous study (B. Guzmán & P. Vargas, unpublished). The mean and standard deviation of the rate of molecular evolution at the ingroup root node (rtrate and rtratesd) was not known a priori. Following the program manual recommendations, rtrate was estimated by calculating the median of the branch lengths from root to ingroup tips, then dividing the median by rttm. For this analysis, the median of branch lengths was 0.003, so rtrate = 0.003/0.32 = 0.009; rtratesd was set equal to rtrate. Brownmean and brownsd were set to 0.4, bigtime was set to 100.0, and commonbrown was set to 0. Other settings were left unchanged. The split age between the *Cistus-Halimium* complex and the *Tuberaria* genus was constrained as upper bound = 0.32. After a burn-in stage of 100,000 cycles (not sampled), the Markov chain was sampled 500,000 times every 100 cycles. We ran this step three times and did not observe any difference.

Lineages-through-time plots (LTT) for the Canarian and the mainland groups were calculated using PL estimated dates and the APE package (Paradis *et al.* 2004) in R software v2.31 (2006) to visualise the number of lineages present (as a proportion of terminals) at sequential time points.

3. Results

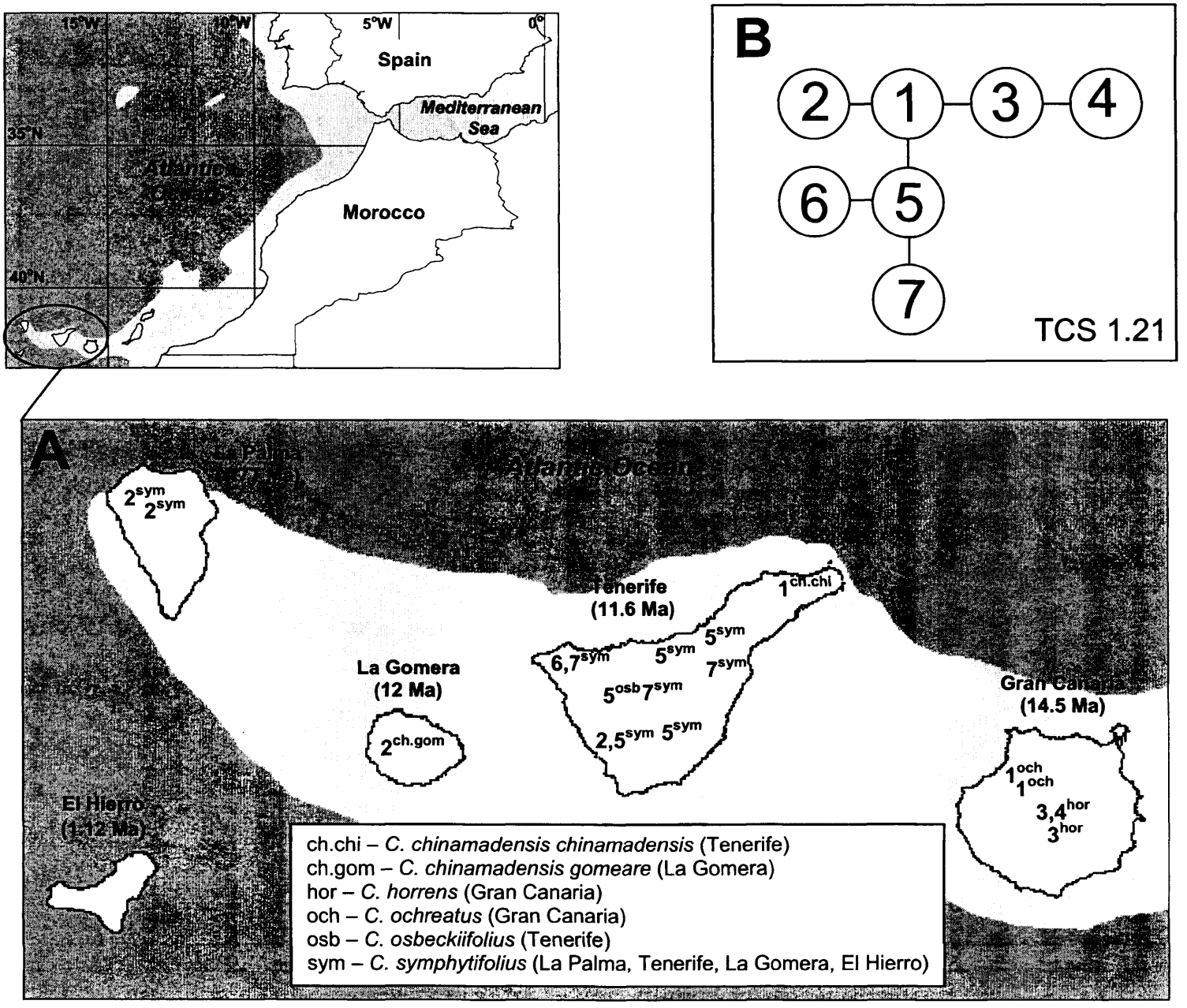
3.1. Analysis of Canarian Cistus *haplotypes*

Sequence length was 1304 bp for *trnK-matK* and 568-590 bp for *trnS-trnG* (Table 2A). The combined data set of *trnS-trnG* and *trnK-matK* sequences (86) distinguished seven

haplotypes (Table 1) distributed in Tenerife (5 haplotypes), Gran Canaria (3 haplotypes), La Gomera (1 haplotype), La Palma (1 haplotype) (Fig. 1A). Haplotypes 1 and 2 were found on different islands (haplotype 1 in Gran Canaria and Tenerife; haplotype 2 in Tenerife, La Palma, La Gomera) and taxa (haplotype 1 in *C. ochreatus* and *C. chinamadensis* subsp. *chinamadensis*, and haplotype 2 in *C. symphytifolius* and *C. chinamadensis* subsp. *gomerae*). Haplotype 5 was shared by *C. osbeckiifolius* and *C. symphytifolius*, while haplotypes 3 and 4 were exclusively found in *C. horrens* (Gran Canaria) and haplotypes 6 and 7 in *C. symphytifolius* populations from Tenerife. In the populations where two or three individuals were sequenced, 12 of 18 (66.66%) samples had a single haplotype (Table 1). Only one population of *C. horrens* (Gran Canaria) and two of *C. symphytifolius* (Tenerife) showed two different haplotypes.

Table 2. List of haplotypes found in 22 populations from five species endemic to the Canary Islands (A) and in 21 populations from three mainland purple-flowered species of *Cistus* (B). Variable sites (excluding indels) in the data sets of two plastid DNA fragments (*trnK-matK*, *trnS-trnG*) are shown. Nucleotide position in each matrix is numbered from the 5′ to the 3′ ends

A. Canary Islands	*trnK-matK*			*trnS-trnG*		
Nucleotide position	352	597	973	153	327	352
Haplotype 1	A	G	A	T	T	A
2	A	G	A	T	A	A
3	A	G	A	G	T	A
4	A	T	A	G	T	A
5	G	G	A	T	T	A
6	G	G	A	T	T	C
7	G	G	G	T	T	A
B. Mainlad						
Nucleotide position	102	497	513	2	79	279	512
Haplotype A	C	G	A	T	C	A	G
B	C	G	A	C	C	A	G
C	A	G	A	T	C	A	G
D	C	G	G	T	C	C	T
E	C	G	A	T	A	A	T
F	C	A	A	T	A	A	T


15°W
10°W
5°W
0°
Spain
Mediterranean Sea
35°N
Atlantic Ocean
Morocco
40°N
B
2
1
3
4
6
5
7
TCS 1.21
A
Atlantic Ocean
2sym 2sym
Tenerife (11.6 Ma)
1ch.chi
5sym
6,7sym
5sym
7sym
5osb 7sym
2,5sym 5sym
La Gomera (12 Ma)
2ch.gom
Gran Canaria (14.5 Ma)
1och 1och
3,4hor
3hor
El Hierro (1.12 Ma)
ch.chi – C. chinamadensis chinamadensis (Tenerife)
ch.gom – C. chinamadensis gomeare (La Gomera)
hor – C. horrens (Gran Canaria)
och – C. ochreatus (Gran Canaria)
osb – C. osbeckiifolius (Tenerife)
sym – C. symphytifolius (La Palma, Tenerife, La Gomera, El Hierro)

TCS constructed a single network with seven haplotypes (Fig. 1B), four haplotype clades and no loops. The interior haplotypes 1 and 5 display the higher number of mutational connections (three connections). Three groups of haplotypes are related to geography. Haplotypes 1, 3, 4 show an eastern distribution (northeast Tenerife, Gran Canaria), haplotypes 5, 6, 7 are restricted to Tenerife and haplotype 2 has a western distribution (La Palma, La Gomera, southwest Tenerife).

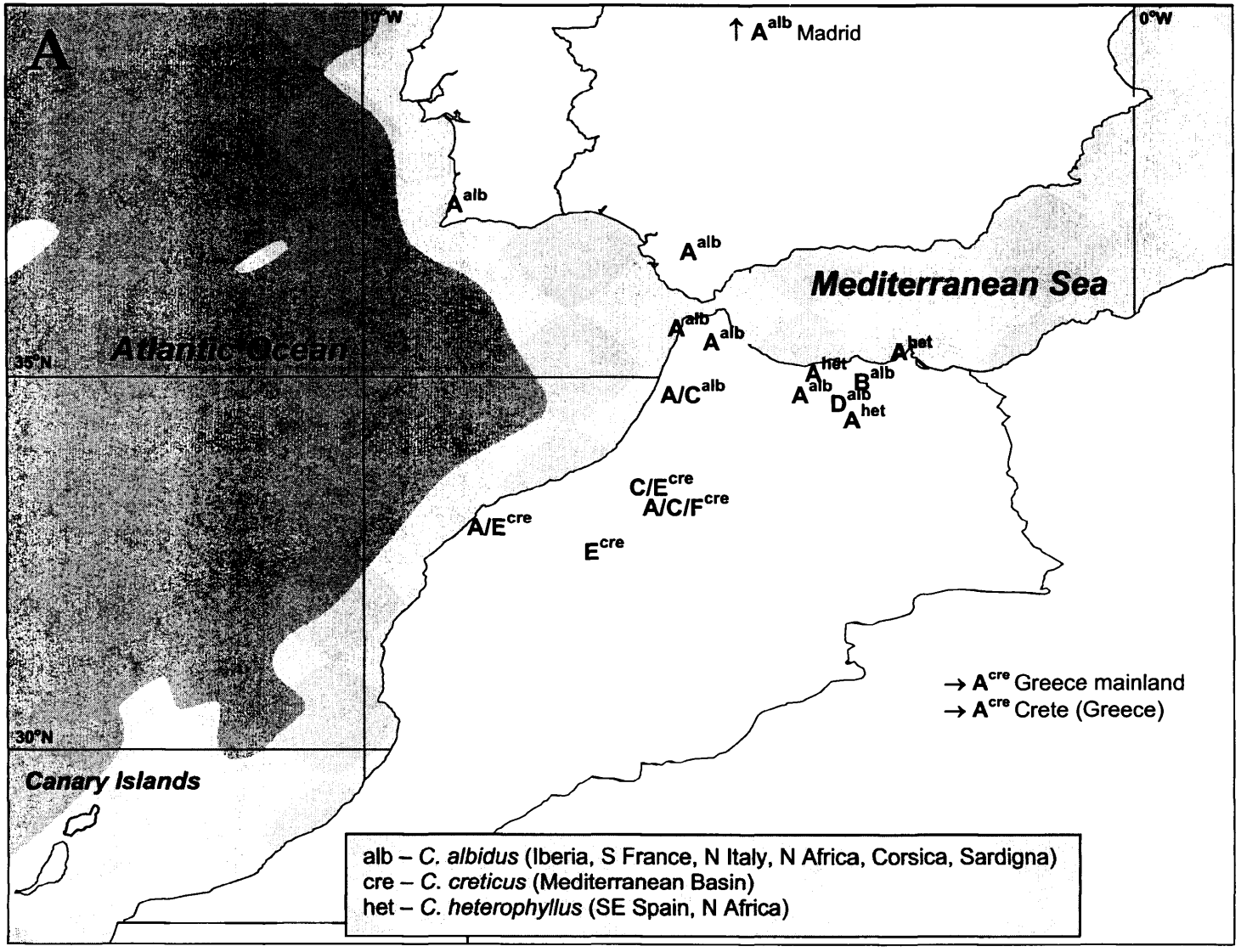
When coding indels (one within the *trnS-trnG* spacer sequences after removing the mononucleotide repeat stretches, poly-A) TCS constructed a network with eight haplotypes (results not shown). A new haplotype (8) distributed in Tenerife was found in two populations of *C. symphytifolius* (one population from Güimar and another one from Teide). Haplotype 8 was connected by one mutational step to haplotype 7 and haplotype 5. We hereafter considered the network analysis with no indel coding for the shake of brevity and accuracy, given cpDNA gaps are highly homoplastic (Clegg *et al.* 1994).

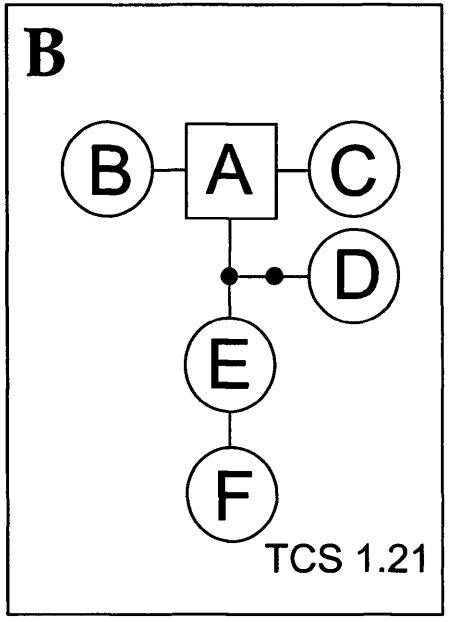
3.2. Analysis of mainland Cistus *haplotypes*

Sequence length of the purple-flowered *Cistus* species from mainland was 1304 bp for *trnK-matK* and 563-603 bp for *trnS-trnG* (Table 2B). The combined data set of *trnS-trnG* and *trnK-matK* sequences (54) distinguished six haplotypes (Table 1), which are distributed in Europe (1 haplotype) and Africa (6 haplotypes) (Fig. 2A). The most common and widespread haplotype (A) was found in 15 individuals (55.55% of all samples) from populations of the two continents. This haplotype accounts for 73% of C. *albidus* individuals, 31% of C. cre*ticus* and 100% of *C. heterophyllus*. One more haplotype

←

Fig. 1. Geographical distribution (A) of the seven cpDNA haplotypes of Canarian *Cistus*. Numbers indicate the seven haplotypes. Statistical parsimony network (B) representing relationships of the seven plastid (*trnS-trnG, trnK-matK*) haplotypes of the five species endemic to the Canary Islands. Lines between haplotypes in the network indicate a single nucleotide substitution.


A
10°W
0°W
↑ A^alb Madrid
A^alb
A^alb
Mediterranean Sea
A^alb
A^alb
A^het
A^het
A^alb
B^alb
A/C^alb
A^alb
D^alb
A^het
C/E^cre
A/C/F^cre
A/E^cre
E^cre
35°N
Atlantic Ocean
30°N
Canary Islands
→ A^cre Greece mainland
→ A^cre Crete (Greece)
alb – C. albidus (Iberia, S France, N Italy, N Africa, Corsica, Sardigna)
cre – C. creticus (Mediterranean Basin)
het – C. heterophyllus (SE Spain, N Africa)


B
B
A
C
D
E
F
TCS 1.21

(C) was recorded across two taxa (*C. albidus, C. creticus*). Haplotypes B and D were exclusively found in African populations of *C. albidus* and haplotypes E and F in African populations of *C. creticus*. We retrieved homogeneity of haplotypes in five of the18 populations. Accordingly, the majority of populations (80%) were not fixed for a particular haplotype (Table 1).

TCS constructed a single network of the six haplotypes, four haplotype clades and no loops (Fig. 2B). The network depicted haplotype A and a missing one (extinct or not found) as the two interior haplotypes with more mutational connections (three connections). Two groups of haplotypes (A-B-C/E-F) were separated by one missing haplotype, which is also connected with haplotype D through another missing haplotype.

When coding indels (five indels within the *trnS-trnG* spacer sequences after removing the mononucleotide repeat stretches, poly-A) TCS constructed a network with ten haplotypes (results not shown). The new haplotypes were distributed as follow: haplotype G in one population of *C. heterophyllus* (Targuist) and one of *C. creticus* (Creta); haplotype H in one population of *C. heterophyllus* (Gurugú Mountain); haplotype I in one population of *C. albidus* (Fardiwa), three populations of *C. creticus* (Creta, Azilal (one individual), Essaouira (one individual)), one population of *C. heterophyllus* (Beni Hadifa); haplotype J in one population of *C. creticus* (one individual from Azilal). We hereafter considered the network analysis with no indel coding for the shake of brevity and accuracy, given cpDNA gaps are highly homoplastic (Clegg *et al.* 1994).

←

Fig. 2. Geographical distribution (A) of the six cpDNA haplotypes of mainland purple-flowered *Cistus* species. Letters indicate the six haplotypes. Statistical parsimony network (B) representing relationships of the six plastid (*trnS-trnG, trnK-matK*) haplotypes of the three mainland purple-flowered species of *Cistus*. Lines between haplotypes in the network indicate a single nucleotide substitution and dots (•) represents missing haplotypes (extinct or not found).

3.3. Phylogenetic analyses

The aligned length of the combined *trnK-matK* and *trnS-trnG* sequences of the *Cistus*, *Halimium* and *Tuberaria* species was 2168 bp. Seventy four of the 249 variable characters were parsimony informative. The MP analysis generated 852 trees of 306 steps with a consistency index (CI) of 0.90, a retention index (RI) of 0.93 and a rescaled consistency index (RC) of 0.84. The strict consensus tree (results not shown) was mostly consistent with the BI tree. The consensus BI tree using the simplest model of evolution (GTR+G) is shown in Figure 3. Both analyses retrieved a mainland purple-flowered species (*C. crispus*) as sister to the group of the Canarian and the mainland purple-flowered clades (100 PP, 99% BS). The BI and MP trees also recognized the Canarian species and the three mainland purple-flowered species (*C. albidus, C. creticus, C. heterophyllus*) as two monophyletic groups (100 PP, 100% BS and 100 PP, 93% BS, respectively). Within the Canarian clade both analyses retrieved the haplotype 2 as sister (94 PP, 63% BS) to the other Canarian haplotypes. Three more haplotype lineages are distributed in Tenerife and Gran Canaria (1), Gran Canaria (3-4) and Tenerife (5-6-7). The five Canarian endemics and the white-flowered species *C. monspeliensis* may have colonized the archipelago in, at least, two independent colonization events, as the species are placed in two different clades (Fig. 3).

Within the mainland purple-flowered clade, MP and BI analyses retrieved the haplotype F as sister to the other haplotypes (92 PP, 57% BS). Three more haplotype lineages (E/D/A-B-C) are primarily distributed in northern Africa, with only haplotype A distributed in other areas.

→

Fig. 3. Chronogram of the consensus Bayesian inference tree based on the combined data sets of *trnS-trnG* and *trnK-matK* sequences. Branch lengths represent million of years (Ma). Values above and bellow branches are posterior probabilities and bootstrap of MP analysis, respectively. One estimated date (node A) of the *Cistus-Halimium* complex divergence (Wikström *et al.* 2001) was used to implement the analysis. Outgroup taxa (*Tuberaria guttata*) have been removed for legibility. Lineages-through-time plots of the Canarian (•) and the purple-flowered mainland (∇) lineages are also shown (inset).

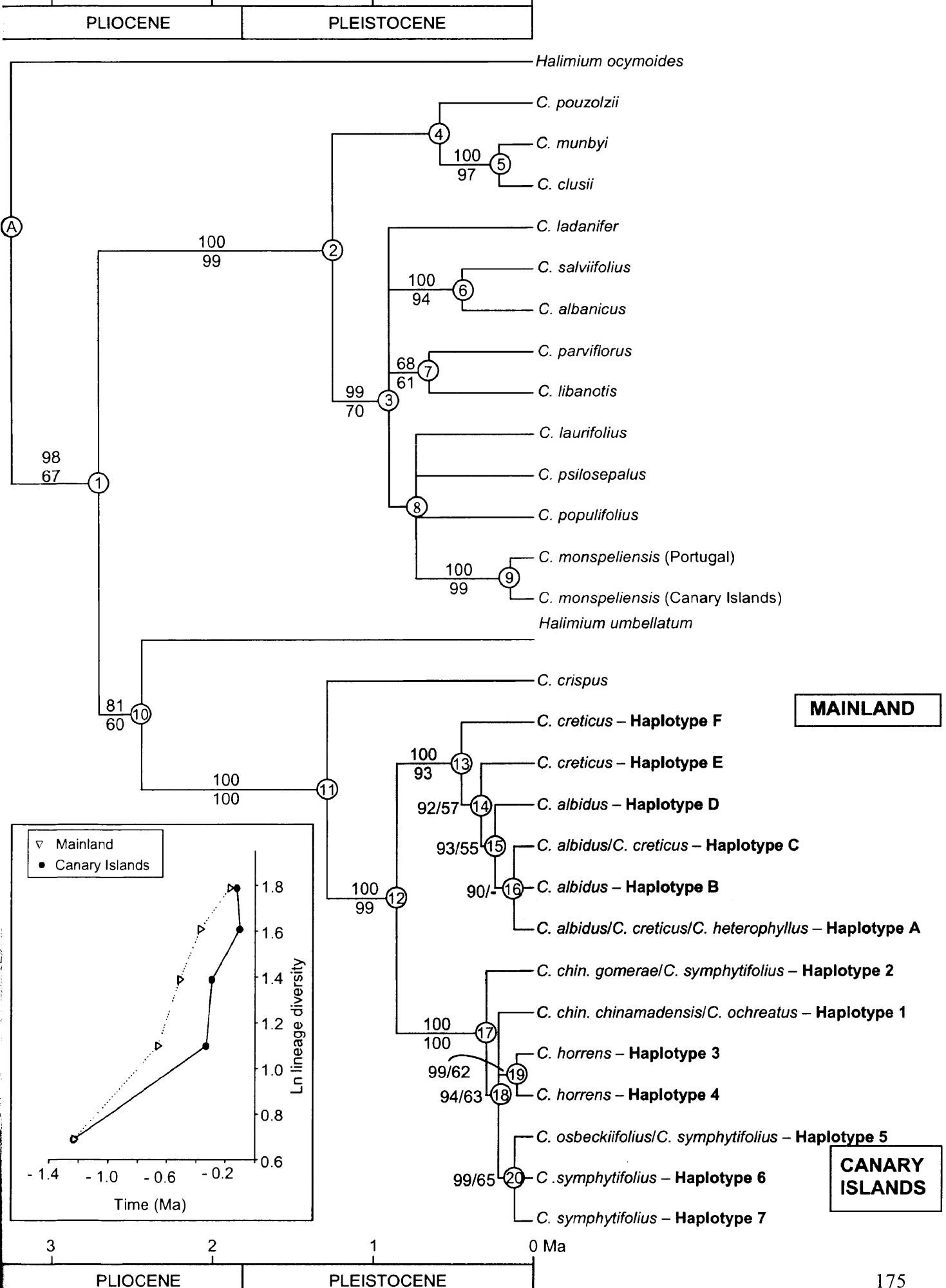

PLIOCENE
PLEISTOCENE
Halimium ocymoides
C. pouzolzii
C. munbyi
C. clusii
C. ladanifer
C. salviifolius
C. albanicus
C. parviflorus
C. libanotis
C. laurifolius
C. psilosepalus
C. populifolius
C. monspeliensis (Portugal)
C. monspeliensis (Canary Islands)
Halimium umbellatum
C. crispus
C. creticus – Haplotype F
C. creticus – Haplotype E
C. albidus – Haplotype D
C. albidus/C. creticus – Haplotype C
C. albidus – Haplotype B
C. albidus/C. creticus/C. heterophyllus – Haplotype A
C. chin. gomerae/C. symphytifolius – Haplotype 2
C. chin. chinamadensis/C. ochreatus – Haplotype 1
C. horrens – Haplotype 3
C. horrens – Haplotype 4
C. osbeckiifolius/C. symphytifolius – Haplotype 5
C .symphytifolius – Haplotype 6
C. symphytifolius – Haplotype 7
MAINLAND
CANARY ISLANDS
A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
100
99
100
97
100
94
68
61
99
70
98
67
81
60
100
100
100
93
92/57
93/55
90/-
100
99
100
100
99/62
94/63
99/65
Mainland
Canary Islands
Ln lineage diversity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
- 1.4
- 1.0
- 0.6
- 0.2
Time (Ma)
3
2
1
0 Ma
PLIOCENE
PLEISTOCENE

Table 3. Penalized Likelihood estimates of divergence rates and pairwise sequence divergences of *trnS-trnG* and *trnK-matK* sequences calculated with five different models of nucleotide evolution for purple-flowered mainland and Canarian lineages of *Cistus*. s/s/yr = nucleotide substitutions per site per year

	Divergence rates (s/s/yr)	**Pairwise sequence divergence (%)**				
		JC	F81	K-2-p	HKY85	GTR
White-flowered lineage	3.67×10^{-9}	0.1-1.19	0.1-1.19	0.1-1.19	0.1-1.19	0.1-1.19
Purple-flowered lineage	6.96×10^{-9}	0.05-1.1	0.05-1.1	0.05-1.1	0.05-1.1	0.05-1.1
Mainland lineage	6.67×10^{-9}	0.05-0.27	0.05-0.27	0.05-0.27	0.05-0.27	0.05-0.27
Canarian lineage	7.99×10^{-9}	0.05-0.21	0.05-0.21	0.05-0.21	0.05-0.21	0.05-0.21

Pairwise sequence divergence between samples of the purple-flowered lineage (including *C. crispus*) were similar (ranging from 0.05 to 1.1%) irrespective of the model of sequence substitution selected (Table 3). The same is true for values within the mainland lineage (ranging from 0.05 to 0.27%) and the Canarian lineage (ranging from 0.05 to 0.21%).

3.4. Divergence times

The three replicate dating runs implementing the Bayesian dating method resulted in identical results, suggesting that all the runs reached stationary. The results of both dating analyses (relaxed Bayesian molecular clock, Penalized Likelihood) are shown in Table 4 and Fig. 4A. The ages estimated for nodes showed a positive linear relationship (Fig. 4B) being the dates estimated with the Penalized Likelihood method older than those estimated with the Bayesian method (Fig. 4A). No major incongruence between ages estimated with both methods has been found. Hereafter we will discuss results retrieved from the Penalized Likelihood approach for the shake of brevity and comparison with other plant groups.

The molecular clock analyses estimated that the divergence of *Halimium umbellatum* and the two major lineages of the genus *Cistus* (one including the purple-flowered species and the other of the white-flowered species plus the pinkish-flowered *C. parviflorus*) occurred in the Middle Pliocene (3.13 ± 0.08 Ma) (node 1 in Table 4; Fig. 4). In the purple-flowered lineage, two sublineage divergences took placed almost simultaneously. An ancestor closely related to *C. crispus* diverged into the mainland lineage (*C. albidus, C. creticus, C. heterophyllus*) about 0.66 ± 0.18 Ma and into the Canarian lineage about 0.33 ± 0.14 Ma (nodes 13 and 17 in Table 4; Fig. 4). PL showed similar local rates per branch (nucleotide substitutions per site per year (s/s/yr)) within the Canarian (7.99×10^{-9} s/s/yr) and the mainland (6.67×10^{-9} s/s/yr) clades while the diversification of the other *Cistus* clade (white-flowered species plus *C. parviflorus*) occurred with a rate of 3.67×10^{-9} s/s/yr (Table 3). The relative rate test showed non significant differences (LRStat = 0.25; d.f. = 5) between the two models tested (constant

and different rates between the clades that are evolving from the same ancestor), so the null hypothesis of constant rates in both clades cannot be rejected.

Table 4. Penalized Likelihood (bootstrapping of 1000 trees) and relaxed Bayesian molecular clock estimates of ages for constrained and unconstrained nodes. The first node (A) is assigned a maximum age (indicated in parentheses) as derived from a previous molecular clock study (B. Guzmán & P. Vargas, unpublished; Wikström *et al.* 2001). The letter and numeric codes for each node of the phylogeny of Cistaceae correspond to those shown in Fig. 3. Ma = million years; SD = standard deviation

	Penalized Likelihood (PL)				**relaxed Bayesian clock**			
Node	Mean age (Ma)	SD (Ma)	Maximum age (Ma)	Minimum age (Ma)	Mean age (Ma)	SD (Ma)	Maximum age (Ma)	Minimum age (Ma)
A (3.26)	3.26	0.00	3.26	3.26	2.4	0.52	3.17	1.23
1	3.13	0.08	3.23	2.78	2.21	0.50	3.03	1.15
2	2.15	0.29	3.02	1.03	1.33	0.41	2.23	0.63
3	1.90	0.28	2.52	1.00	1.08	0.34	1.85	0.50
4	1.19	0.48	2.59	0.14	0.91	0.36	1.70	0.31
5	0.26	0.22	2.27	0.004	0.26	0.19	0.72	0.01
6	0.90	0.29	2.01	0.14	0.47	0.27	1.12	0.06
7	1.51	0.41	2.74	0.0004	0.75	0.30	1.43	0.27
8	1.69	0.31	2.72	0.58	0.88	0.30	1.56	0.39
9	0.25	0.21	1.58	0.004	0.21	0.16	0.61	0.01
10	3.03	0.13	3.23	2.57	2.02	0.49	2.86	1.03
11	1.78	0.28	2.61	0.96	1.15	0.41	2.07	0.46
12	1.23	0.25	1.95	0.51	0.68	0.31	1.41	0.22
13	0.66	0.18	1.42	0.16	0.38	0.19	0.83	0.11
14	0.51	0.16	1.15	0.09	0.27	0.15	0.64	0.06
15	0.37	0.14	0.82	0.03	0.19	0.12	0.50	0.03
16	0.17	0.10	0.49	0.004	0.10	0.09	0.33	0.003
17	0.33	0.14	0.88	0.07	0.28	0.16	0.68	0.07
18	0.29	0.11	0.65	0.05	0.17	0.11	0.46	0.02
19	0.10	0.09	0.59	0.004	0.08	0.08	0.28	0.002
20	0.12	0.08	0.49	0.004	0.08	0.08	0.29	0.002

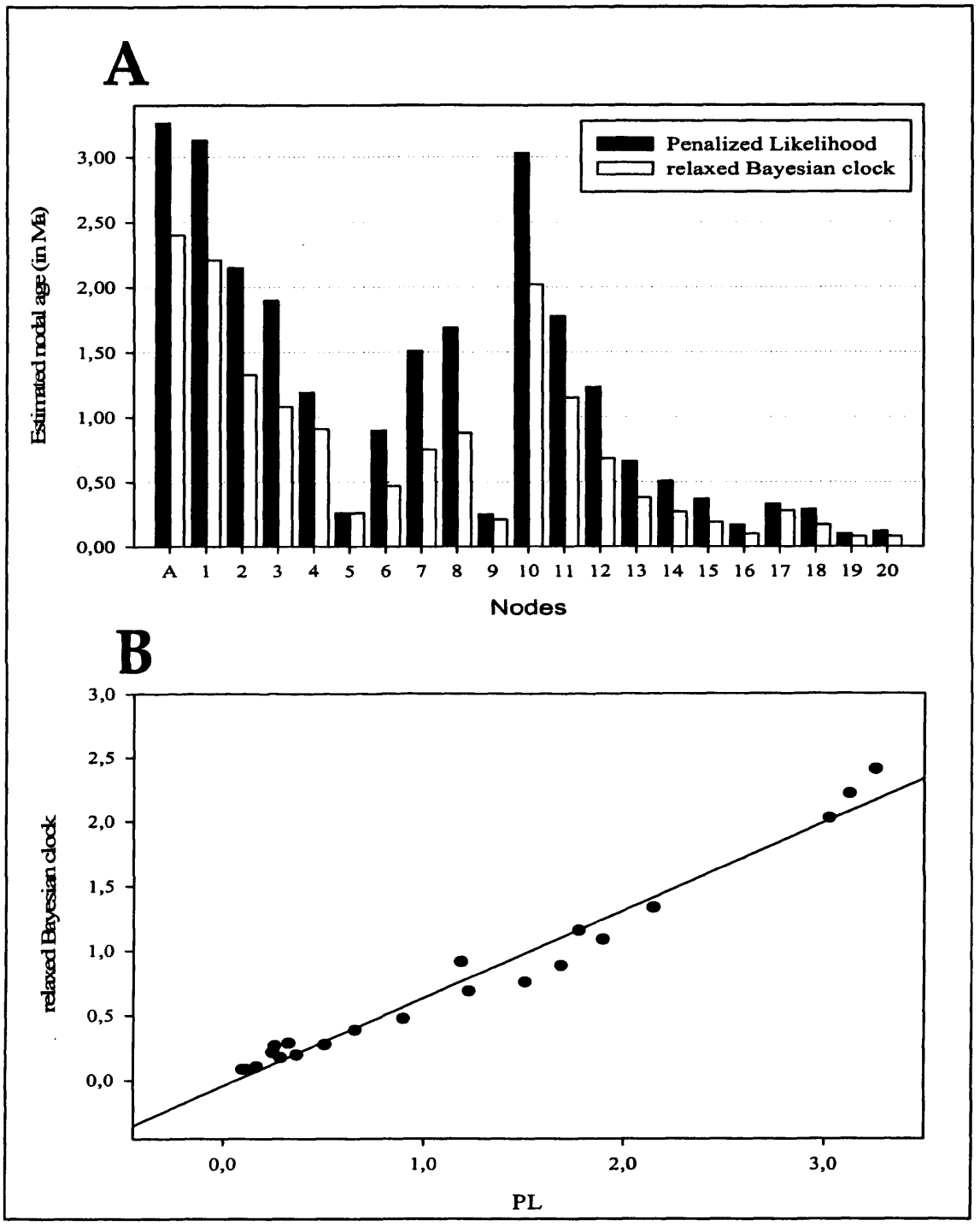


Fig. 4. Descriptive statistical comparisons of mean nodal ages derived from the *trnK-matK/trnS-trnG* data set (See Table 4). (a) Comparison of age estimates derived from the Penalized Likelihood (PL, bootstrapping of 1000 trees) and the relaxed Bayesian molecular clock approaches. Ma = million years. (b) PL estimates are compared with those derived from the relaxed Bayesian molecular clock approach.

The lineages-through-time plots for Canarian and mainland lineages are shown in the inset of Fig 3. The shape of the plots hints at a comparable behaviour in both sister groups. Our analysis reflects that since after a long period of stasis (570,000 and 900,000 years in the mainland and Canarian lineages, respectively) the same number of haplotype lineages (4) was generated in both groups.

4. Discussion

4.1. Synchronous processes of radiation in Canarian and mainland lineages

All phylogenetic analyses are congruent with a sister group relationship of the three mainland (*C. albidus, C. creticus, C. heterophyllus*) and the five Canarian (*C. chinamadensis, C. horrens, C. ochreatus, C. osbeckiifolius, C. symphytifolius*) species of purple-flowered *Cistus* (Fig. 3). Levels of diversity in species number (3 vs. 5) in the mainland and the Canary Islands are comparable to those of genetic diversity. Despite purple-flowered *Cistus* is widely distributed in northern Africa and the Iberian Peninsula, but more restricted in the Canary Islands, similar number of haplotypes (7 vs. 6) and the same number of haplotype clades (4) suggest a case of synchronous differentiation (Fig. 1, 2, 3).

This Canarian-Mediterranean group displays a moderate differentiation into eight purple-flowered species, which appeared to have undergone rapid and recent differentiation as suggested by the maximum sequence divergences of 1.1 % (Table 3). Our molecular clock is congruent with an estimated age of 1.23 ± 0.25 Ma (Table 4) after the split of the most common recent ancestor with *C. crispus* (Fig. 3). The question remains whether insular species are the result of a faster differentiation process with respect to mainland relatives.

A pattern of faster evolution on the Canarian islands with respect to mainland groups have been described on the basis of molecular data assuming a clock-like average constancy of base-pairs substitutions (Böhle *et al.* 1996; Kim *et al.* 1996; Krupkin *et al.* 1996; Francisco-Ortega *et al.* 1997; Baldwin *et al.* 1998; Francisco-Ortega *et al.* 1999; Thiv *et al.* 1999; Caujapé-Castells *et al.* 2001). The much higher maximum sequence

divergence found within mainland species regarding to their insular relatives has been explained on the basis of the faster evolution occurred on islands (e.g. 0.31% - 0.61% in the silversword alliance-Californian tarweeds; 0.15% - 0.29% in *Argyranthemum*-mainland relatives; Francisco-Ortega *et al.* 1996). In our study case, a synchronous processes of evolution is in contrast suggested by the similarity of maximum pairwise sequence divergences found in the Canarian and the mainland lineages (0.21% and 0.27%, respectively) (Table 3).

As demonstrated elsewhere, there is no universal clock and rates of evolution can vary also between closely related lineages (Sanderson 2002). Although we rejected the hypothesis of rate constancy among lineages of *Cistus* (χ^2 = 175.64; d.f. = 29), a constant rate of molecular evolution occurred since the split of the mainland and the Canarian lineages (LRStat = 0.25; d.f. = 5). In particular, an early stage of radiation is suggested by estimated ages of 0.66 ± 0.18 Ma in the Mediterranean and 0.33 ± 0.14 Ma in the Canary Islands. This result supports one more aspect of the hypothesis of synchronous differentiation: similar differentiation times between lineages (Fig. 3).

The divergence of *Halimium umbellatum*, the purple- and the white-flowered *Cistus* lineages (3.13 ± 0.08) coincides with the onset (3.2 Ma) and establishment (2.8 Ma) of the Mediterranean climate (Suc *et al.* 1995). When the mainland and the Canarian purple-flowered lineages split, synchronous radiation occurred with the advent of the Pleistocene (< 1.8 Ma), as revealed by the *trnK-matK/trnS-trnG* chronogram (Fig. 3, Table 4). No evidence of a significant accelerated rate of speciation is found for the insular group. Relatively rapid radiation occurred in mainland lineages of *Cistus* not only in the purple-flowered group but also in the white-flowered lineage (2.15 ± 0.29; see node 2 of Table 4, Fig. 3). Establishment of the Mediterranean climate (2.8 Ma, Suc *et al.* 1995) may have been responsible to trigger rapid speciation in mainland. We hypothesize that no significant difference is observed between mainland and island radiations because the two areas conform two hotspots of rapid differentiation (Myers *et al.* 2000).

4.2. Testing adaptive radiation in Canarian Cistus

Adaptive radiation is the evolution of ecological and phenotypic diversity within a rapidly multiplying lineage that may be detected by four features: (1) common ancestry, (2) rapid speciation, (3) trait utility, (4) phenotype-environment correlation (Schluter 2003).

Common ancestry of Canarian *Cistus* is clearly documented from our results. The phylogenetic analysis (Fig. 3) provides a strong support for the monophyly of the Canarian *Cistus*, in agreement with previous nuclear and plastid phylogenies (Guzmán & Vargas 2005). This is consistent with the pattern found in other Macaronesian plant groups (*Echium*, Böhle *et al.* 1996; *Argyranthemum*, Francisco-Ortega *et al.* 1996; *Sonchus* alliance, Kim *et al.* 1996; *Pericallis*, Panero *et al.* 1999; but not in others, Vargas 2007) and implies a single mainland to island invasion event in the history of the Canarian purple-flowered *Cistus*.

Relatively rapid speciation in Canarian *Cistus* is interpreted by the number of species (5) originated in a short period of time (330.000 ± 140.000 years). The most remarkable examples of plant radiation in oceanic islands (Hughes & Eastwood 2006) appear to parallel this figure: 25 species of the silversword alliance in 5.5 ± 0.3 Ma (Baldwin & Sanderson 1998); eight species of *Echium* generated in 1.85 ± 0.5 Ma (Böhle *et al.* 1996; García-Maroto et al., unplublished).

Utility of particular reproductive traits is found in Canarian endemics. Island and mainland populations of plants often differ in their reproductive biology because altered pollination conditions have influenced the floral biology and mating systems of island plants (Sakai *et al.* 1997; Barrett 1998b). Many of the *Cistus* species are self-sterile but Canarian *Cistus* endemics are self-compatible (Warburg & Warburg 1930). This condition promotes colonization as only one immigrant is sufficient to establish a new population (Baker 1955). One additional mechanism promotes heterozygosity by separating spatially anthers and stigmas within a flower (hercogamy) (Barrett 1998a). It is remarkable that the only species of *Cistus* with styles longer than stamens are found

in the Canary Islands. The common ancestor of the Canarian and the mainland purple-flowered lineages appeared to have a style equal in length to the stamens (based on the hypothesis of character evolution for style length presented in Guzmán & Vargas (2005)). By the time the *Cistus* ancestor colonized the Canary Islands this reproductive feature was maintained in the mainland lineage and shifted in the Canarian lineage. A strong trend to separation of flower sex parts has been documented in up to 20% of oceanic insular floras (Sakai *et al.* 1997).

Phenotype-environment correlation is hardly observed in *Cistus*. Some species occur in particular habitats: *C. chinamadensis* in laurisilva forests of Anaga and central area of La Gomera; *C. horrens* in pine forest of southern Gran Canaria; *C. ochreatus* in pine forests of Tamadaba; *C. osbeckiifolius* in high mountain habitats of Tenerife; *C. symphytifolius* in a wide range of habitats of Tenerife and La Palma. The phylogeographic (Fig. 1A) and the phylogenetic (Fig. 3) analyses are not congruent with a relationship between phylogeny and ecology in *Cistus* as several species inhabiting different habitats share the same haplotype and different haplotypes are found in the same species (Table 1 and Fig. 1A). Several studies of large groups of Macaronesian plants have, in contrast, found a correlation between phylogeny and ecology, which was used to describe a pattern of diversification via inter-island colonization between similar ecological zones (Francisco-Ortega *et al.* 1996; Kim *et al.* 1996; but see Percy & Cronk 2002). We consider that a limited period of time precluded diversification of Canarian *Cistus* giving us the opportunity to witness a relatively early process of radiation. The fact that *C. symphytifolius* has a widespread distribution (Fig 1A), shares the greatest genetic divergence (also *C. horrens*), forms part of different lineages (Fig. 1A, 3) and displays the highest allozymic diversity (Batista *et al.* 2001) leads us to hypothesize an early divergence of *C. symphytifolius* (or an ancestor) to bring about four more species by geographic isolation. In particular, Tenerife may have served as centre of dispersal for the Canarian *Cistus* species as it is the island with the highest number of haplotypes (5 haplotypes) and species (*C. chinamadensis* subsp. *chinamadensis, Cistus osbeckiifolius, Cistus symphytifolius*) (Fig. 1A). Other studies (Francisco-Ortega *et al.* 2002;

Allan *et al.* 2004) stressed the historical role of Tenerife as a centre of dispersal in the Canary Islands. From Tenerife, inter-island colonization eastwards and westwards following a geographic stepping stone, rather than ecological, model is the most plausible scenario. In fact a geographic correspondence with haplotype distribution has been detected (Fig. 1A): haplotype 1 was found in the east coast of Tenerife and Gran Canaria; haplotype 2 was found in the southwest Tenerife, La Gomera and La Palma.

Spectacular radiations occurred in some island plant groups can be explained due to both the existence of uncolonized environments and interspecific competition for resources (e.g., 30 species of *Echium*, 28 species in the Hawaiian silversword alliance, 23 species of *Argyranthemum*, 24 species of *Sideritis*). A *Cistus* ancestor reached the islands considerable later (0.33 ± 0.14 Ma) after island formation (c. 20 Ma for Fuerteventura) and underwent a relatively modest radiation (five species). Ecological opportunities in oceanic islands, that increase the survival and establishment of the new immigrant species, diminished when habitats had been occupied by early species (niche pre-emption hypothesis; MacArthur & Wilson 1967; Silvertown 2004). *Cistus* may have had to cope with major establishment limitations in the Canary islands because: (1) the abundance of *Cistus* in the insular vegetation is extremely limited compared to the important role played in the formation of the Mediterranean scrub; (2) LLT plots (inset in Fig. 3) showed that diversification occurred (0.33 ± 0.14 Ma) significantly after estimates of ancestor arrival (1.23 ± 0.25 Ma) to the Canary Islands and then a period of diversification stasis is interpreted. In summary, Canarian *Cistus* appears to have undergone geographic rather than habitat-dependent radiation. Given that dispersal is a faster process than phenotype-environment differentiation and that several haplotypes and species are related to particular habitats, the question remains whether adaptive radiation will take place in *Cistus* over time.

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References

Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap? a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**, 255-266.

Allan GJ, Francisco-Ortega J, Santos-Guerra A, Boerner E, Zimmer EA (2004) Molecular phylogenetic evidence for the geographic origin and classification of Canary Island *Lotus* (Fabaceae: Loteae). *Molecular Phylogenetics and Evolution* **32**, 123-138.

Arrington JM, Kubitzki K (2003) Cistaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 62-70. Springer, Berlin.

Baker HG (1955) Self-compatibility and establishment after long-distance dispersal. *Evolution* **9**, 347-348.

Baldwin BG, Crawford DJ, Francisco-Ortega J, *et al.* (1998) Molecular phylogenetics insights on the origin and evolution of oceanic islands plants. In: *Molecular systematics of plants II. DNA sequencing* (eds. Soltis DE, *et al.*), pp. 410-441. Kluwer Academic Publishers, Boston/Dordretch/London.

Baldwin BG, Robichaux RH (1995) Historical biogeography and ecology of the Hawaiian silverswoord alliance (Asteraceae): new molecular phylogenetic perspectives. In: *Hawaiian biogeography: evolution on a hotspot archipelago* (eds. Wagner WL, Funk VA), pp. 257-287. Smithsonian Institution Press, Washington, D.C.

Baldwin BG, Sanderson MJ (1998) Age and rate of diversification of the Hawaiian silverword alliance (Compositae). *Proceedings of the National Academy of Sciences* **95**, 9402-9406.

Barrett SCH (1998a) The evolution of mating strategies in flowering plants. *Trends in Plant Science* **3**, 335-341.

Barrett SCH (1998b) The reproductive biology and genetics of island plants. In: *Evolution on islands* (ed. Grant PR), pp. 18-34. Oxford University Press, New York.

Batista F, Bañares A, Caujapé-Castells J, *et al.* (2001) Allozyme diversity in three endemic species of *Cistus* (Cistaceae) from the Canary Islands: intraspecific and interspecific comparison and implications for genetic conservation. *American Journal of Botany* **88**, 1582-1592.

Böhle U-R, Hilger HH, Martin WF (1996) Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). **93**, 11740-11745.

Carine MA (2005) Spatio-temporal relationships of the Macaronesian endemic flora: a relictual series or window of opportunity? *Taxon* **54**, 895-903.

Carlquist S (1965) *Island life* Natural History Press, New York.

Carlquist S (1974) *Island biology* Columbia University Press, New York.

Carracedo JC, Pérez FJ, Ancochea E, *et al.* (2002) Cenozoic volcanism II: the Canarian Islands. In: *The Geology of Spain* (eds. Gibbons W, Moreno T), pp. 439-472. The Geological Society of London, London.

Caujapé-Castells J, Jansen RK, Membrives N, *et al.* (2001) Historical biogeography of *Androcybium* Willd. (Colchicaceae) in Africa: evidence from cpDNA RFLPs. *Botanical Journal of the Linnean Society* **136**, 379-392.

Clegg MT, Gaut BS, Learn GH, Morton BR (1994) Rates and patterns of chloroplast DNA evolution. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 6795-6801.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Crawford DJ, Stuessy TF (1997) Plant speciation on oceanic
islands. In: *Evolution and diversification in land plants* (eds. Iwatsuki K, Raven PH), pp. 249-267. Springer-Verlag, Tokyo,
Japan.

Darwin C (1859) *The Origin of Species by Means of Natural Selection,* London.

Francisco-Ortega J, Fuertes-Aguilar J, Kim S-C, *et al.* (2002) Phylogeny of the Macaronesian endemic *Crambe* section *Dendrocrambe* (Brassicaceae) based on internal transcribed spacer squences of nuclear ribosomal DNA. *American Journal of Botany* **89**, 1984-1990.

Francisco-Ortega J, Fuertes Aguilar J, Gómez Campo C, Santos-Guerra A, Jansen RK (1999) Internal transcribed spacer sequence phylogeny of *Crambe* L. (Brassicaceae): molecular data reveal two Old World disjunctions. *Molecular Phylogenetics and Evolution* **11**, 361-380.

Francisco-Ortega J, Jansen RK, Santos-Guerra A (1996) Chloroplast DNA evidence of colonization, adaptive radiation, and hybridization in the evolution of the Macaronesian flora. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 4085-4090.

Francisco-Ortega J, Santos-Guerra A, Hines A, Jansen RK (1997) Molecular evidence for Mediterranean origin of the Macaronesian endemic genus *Argyranthemum* (Asteraceae). *American Journal of Botany* **84**, 1595-1613.

Givnish TJ, Knox E, Patterson TB, *et al.* (1996) The Hawaiian lobelioids are monophyletic and underwent a rapid initial radiation roughly 15 million years ago. *American Journal of Botany (suppl.)* **83**, 159.

Grant PR (1998) Epilogue and questions. In: *Evolution on islands* (ed. Grant PR), pp. 305-319. Oxford University Press, New York.

Guzmán B, Vargas P (2005) Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trn*L-*trn*F, and *mat*K sequences. *Molecular Phylogenetics and Evolution* **37**, 644-660.

Haeckel E (1866) *Generelle Morphologie der Organismen* Reimer, Berlin.

Hamilton M (1999) Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**, 521-523.

Hughes C, Eastwood R (2006) Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences* **103**, 10334-10339.

Huxley J (1942) *Evolution: the modern synthesis* Harper and brothers, New York.

Johnson LA, Soltis DE (1994) *matK* DNA and phylogenetic reconstruction in Saxifragaceae s. str. *Systematic Botany* **19**, 143-156.

Kim S-C, Crawford DJ, Francisco-Ortega J, Santos-Guerra A (1996) A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: evidence for extensive radiation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7743-7748.

Kishino H, Hasegawa M (1989) Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *Journal of Molecular Evolution* **29**, 170-179.

Kishino H, Thorne JL, Bruno WJ (2001) Performance of a divergence time estimation method under a probabilistic model of rate evolution. *Molecular Biology and Evolution* **18**, 352-361.

Krupkin AB, Liston A, Strauss SH (1996) Phylogenetic analysis of the hard pines (*Pinus* subgenus *Pinus*, Pinaceae) from chloroplast DNA restriction site analysis. *American Journal of Botany* **83**, 489-498.

Lamarck JB (1809) *Philosophie zoologique, ou exposition relatives à l'histoire naturelle des animaux* Dentu, Paris.

MacArthur RH, Wilson EO (1967) *The theory of island biogeography* Princeton University Press, Princeton, New Jersey.

Magallón SA, Sanderson MJ (2005) Angiosperm divergence times: The effect of genes, codon positions, and time constraints. *Evolution* **59**, 1653-1670.

Myers N, Mittermeier RA, Mittermeier CG, Fonsaeca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature* **403**, 853-858.

Nylander JAA (2002) MrModeltest v1.0b. Department of Systematic Zoology. Uppsala University, Uppsala.

Osborn HF (1902) The law of adaptive radiation. *The American naturalist* **36**, 353-363.

Panero JL, Francisco-Ortega J, Jansen RK, Santos-Guerra A (1999) Molecular evidence for multiple origins of woodiness and a New World biogeographic connection of the Macaronesian island endemic *Pericallis* (Asteraceae: Senecioneae). *Proceedings of the National Academy of Sciences* **96**, 13886-13891.

Paradis E, Claude J, Strimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R language.

Percy DM, Cronk QCB (2002) Different fates of island brooms: contrasting evolution in *Adenocarpus, Genista,* and *Teline* (Genisteae, Fabaceae) in the Canary Islands and Madeira. *American Journal of Botany* **89**, 854-864.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Provan J, Wayne P, Hollingsworth PM (2001) Chloroplast microsatellites: new tools for studies in plant ecology and evolution. *Trends in Ecology & Evolution* **16**, 142-147.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**, 1572-1574.

Rutschmann F (2005) Bayesian molecular dating using PAML/Multidivtime. A step-by-step manual.

Sakai AK, Weller SG, Wagner WL, Soltis PS, Soltis DE (1997) Phylogenetic perspectives on the evolution of dioecy: adaptive radiation in the endemic Hawaiian genera *Schiedea* and *Alsinidendron* (Caryophyllaceae: Alsinoideae). In: *Molecular evolution and adaptive radiation* (eds. Givnish TJ, Sytsma KJ), pp. 455-473. Cambridge University Press, Melbourne, Australia.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution* **19**, 101-109.

Sanderson MJ, Doyle JA (2001) Sources of error and confidence intervals in estimating the age of angiosperms from *rbcL* and 18S rDNA data. *American Journal of Botany* **88**, 1499-1516.

Sang T, Crawford DJ, Kim SC, Stuessy TF (1994) Radiation of the endemic genus *Dendroseris* (Asteraceae) on the Juan Fernández Islands - evidence from sequences of the ITS regions of nuclear ribosomal DNA. *American Journal of Botany* **81**, 1494-1501.

Santos-Guerra A (1999) Origen y evolución de la flora de Canarias. In: *Ecología y cultura en Canarias* (eds. Fernández-Palacios JM, *et al.*), pp. 107-129. Organismo Autónomo de Museos y Centros-Cabildo de Tenerife, Santa Cruz de Tenerife.

Schluter D (2003) *The ecology of adaptive radiation* Oxford University Press Inc., New York.

Silvertown J (2004) The ghost of competition past in the phylogeny of island endemic plants. *Journal of Ecology* **92**, 168-173.

Simpson GG (1953) *The major features of evolution* Columbia University Press, New York.

Suc JP, Bertini A, Combourieu-Nebout N, *et al.* (1995) Structure of West Mediterranean vegetation and climate since 5.3 Ma. *Acta Zoologica Cracoviensia* **38**, 3-16.

Swofford D (2002) *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.* Sinauer, Sunderland, MA.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619-633.

Thiv M, Struwe L, Kadereit JW (1999) The phylogenetic relationships and evolution of the Canarian laurel forest endemic *Ixanthus viscosus* (Aiton) Griseb. (Gentianaceae): evidence from *matK* and ITS sequences, and floral morphology and anatomy. *Plant Systematics and Evolution* **218**, 299-317.

Thorne JL, Kishino H (2002) Divergence time and evolutionary rate estimation with multilocus data. *Systematic Biology* **51**, 689-702.

Thorne JL, Kishino H, Painter IS (1998) Estimating the rate of evolution of the rate of molecular evolution. *Molecular Biology and Evolution* **15**, 1647-1657.

Vargas P (2007) Are Macaronesian islands refugia of relict plant lineages?: a molecular survey. In: *Phylogeography of Southern European Refugia* (eds. Weiss S, Ferrand N), pp. 297-314. Springer, Dordrecht.

Warburg O, Warburg EF (1930) *A preliminary study of the genus Cistus* Royal Horticultural Society, London.

Wikström N, Savolainen V, Chase MW (2001) Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society of London* **268**, 1-10.

Yang Z (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. *CABIOS* **13**, 555-556 (http://abacus.gene.ucl.ac.uk/software/paml.html).

6

Historical colonization of Mediterranean *Cistus* in spite of unassisted diaspore dispersal

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Abstract

The Mediterranean Basin is a worldwide hotspot for plant diversity and reconstruction of historical processes are needed to understand plant colonization and lineage origin. We studied the 21 species of *Cistus* and a specific sample of 48 populations of the western Mediterranean *C. ladanifer* by means of sequence analysis of three (*rbcL, trnK-matK, trnS-G*) plastid data sets. A phylogeographical study of 112 *C. ladanifer* sequences of the *trnS-trnG* and *trnK-matK* spacers revealed eight nucleotide haplotypes significantly distributed on the European (4) and African (5) sides of the Strait of Gibraltar. Both the haplotype network and phylogenetic analysis depicted two primarily European (subspp. *sulcatus, ladanifer*) and African (subspp. *africanus, ladanifer*) lineages. At least two intercontinental colonizations are interpreted as a result of disassociation between geographical and lineage distributions of haplotypes and timing estimates. Occurrence of a tip haplotype from southern Iberia in the African lineage supports the hypothesis of long-distance dispersal rather than vicariance. Molecular clock estimates of two plastid (*rbcL, trnK-matK*) and one nuclear (ITS) data sets are fully consistent with a second event of long-distance dispersal of an interior haplotype of *C. ladanifer* significantly postdating the opening of the Strait of Gibraltar (c. 5 Ma). Phylogenetic, phylogeographical, molecular clock, paleobotanical, ecological and distributional results lead us to interpret a long history of active long-distance dispersal of *Cistus* across the Mediterranean coupled with predominant leading edge expansion. We argue that, despite limited dispersal abilities, preference to xeric habitats has been decisive to historical colonization after the advent of the Mediterranean climate (c. 3.2 Ma), when *Cistus* species early became part of the dominant element in the Mediterranean scrub.

Key words: Cistaceae, *Cistus*, ITS, molecular clock, phylogeography, *rbcL*, Strait of Gibraltar, *trnS-trnG*, *trnK-matK*, unassisted dispersal

1. Introduction

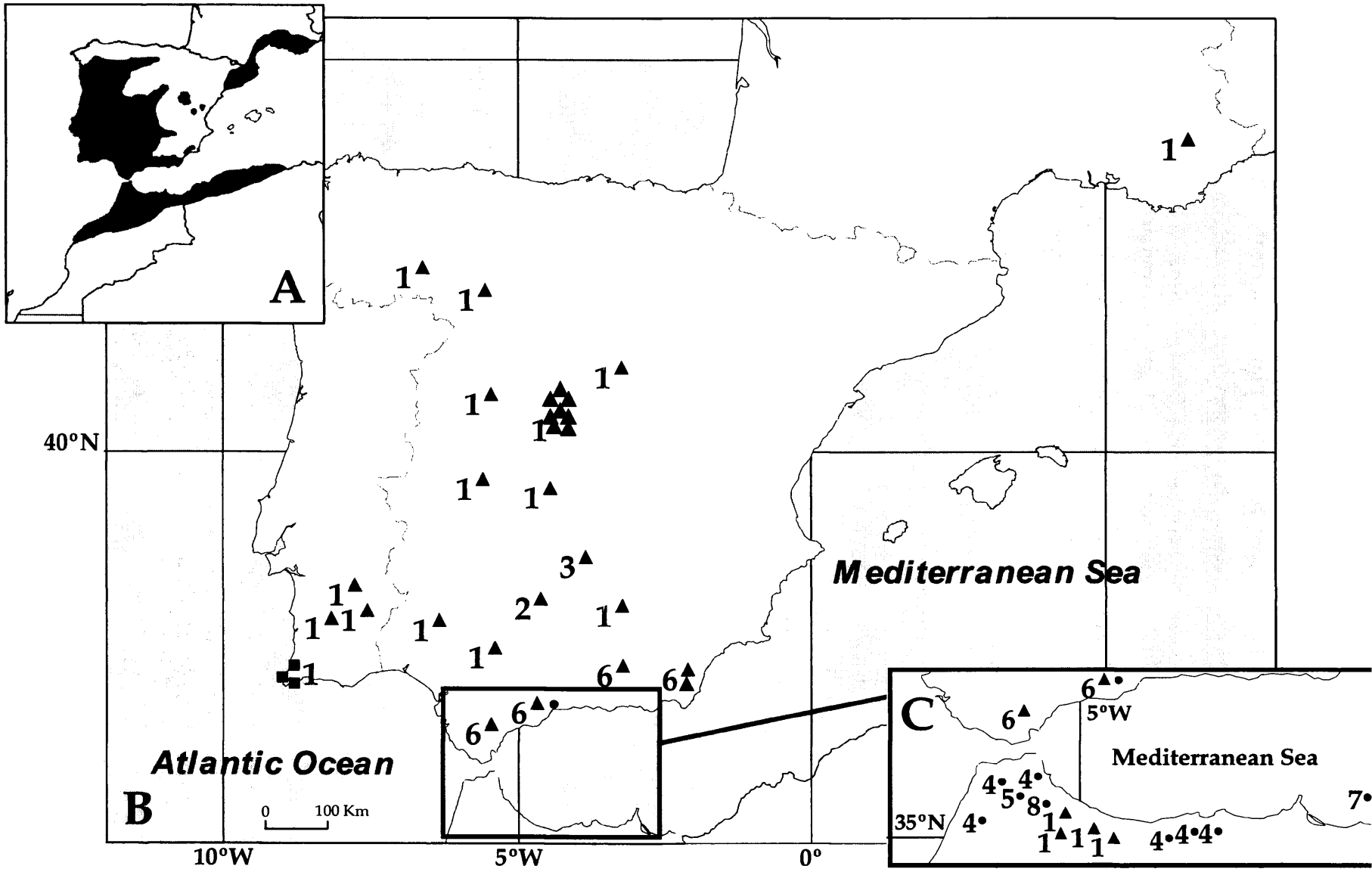
The Mediterranean Basin harbours more than 25,000 plant species, being more than half endemics (Blondel & Aronson 1999), and 10 of the 34 hotspots on Earth (Mittermeier *et al.* 2004). Médail & Quezel (1997) considered two major centres of biodiversity in the Mediterranean Basin: the western (including Iberian Peninsula and Morocco) and the eastern (Turkey and Greece) areas. In fact, the Mediterranean region of Iberia contributes with 5000 species (of which 19.1% are endemic), while the Mediterranean region of Morocco displays less alpha diversity (3800 species) but higher percentage of endemism (21.4%) (Médail & Quezel 1997). The complexity of factors such as biogeography, geology, paleoclimatology and ecogeographical heterogeneity accounts for the high levels of biodiversity and endemism found in the Western Mediterranean. Despite being a hotspot with important levels of species diversity, the origin and patterns of evolution of Mediterranean populations are poorly understood (Vargas *et al.* 1999; Caujapé-Castells *et al.* 2001; Coleman *et al.* 2003; Pérez *et al.* 2004; Albaladejo *et al.* 2005). Taking into consideration alpha diversity as an indicator of intraspecific lineage diversity, complex evolutionary patterns at the population level are also expected and phylogeographical studies needed to infer early stages of species differentiation.

One of the most remarkable gene flow barriers in the Western Mediterranean is the Strait of Gibraltar, which separates the Iberian Peninsula from northern Africa by 14.4 Km of open sea between the closest points. Dispersal of plants appears to have been hindered by the Strait of Gibraltar since origin (c. 5 Ma) because of absence of 25% of the 3500 angiosperm species distributed on both sides (Valdés 1991). During the Messinian Salinity Crisis (5.96-5.33 Ma, Duggen *et al.* 2003), when the Mediterranean Sea desiccated, land bridges facilitated range expansion of plants with no syndrome for long-distance dispersal (Vargas *et al.* 1999; Caujapé-Castells & Jansen 2003). The opening of the Strait of Gibraltar interrupted land connection between Europe and Africa. Since then, plant distribution have fully depended on long-distance dispersal, although periodical emergence of islands in the Western Mediterranean region during

Quaternary glaciations could have facilitated plant dispersal between the Iberian Peninsula and Northern Morocco (Ortiz *et al.* 2007).

The genus *Cistus* L. (Cistaceae) comprises 21 species, primarily distributed in the Mediterranean region. *Cistus* species are characterized by a relatively high number (500-18,000) of medium-size (1-2 mm) seeds enclosed in 6 (-12) carpels of a dry, loculicidal capsule. Given the five major groups of diaspore syndromes for dispersal (Van der Pijl 1979), we classify not only *Cistus* but also the Cistaceae species in the group of angiosperms lacking structures for long-distance dispersal (Herrera 1992; Malo & Suárez 1996; Bastida & Talavera 2002). Distribution patterns of Mediterranean species points to *Cistus* as a suitable plant group to study historical colonizations in spite of unassisted diaspore dispersal. Few species are endemic to particular countries and three species of different groups display a circum-Mediterranean distribution (*C. creticus, C. monspeliensis, C. salviifolius*). In addition, five species of *Cistus* are endemic to the Canary Islands, which successfully colonized five western islands from a single purple-flowered ancestor (Guzmán & Vargas 2005). In the small region of the Western Mediterranean, 14 of the 21 *Cistus* species occur in the area around the Strait of Gibraltar. Plant distribution between the two unconnected parts of Western Europe and Africa in the last 5 million years offers the opportunity to test whether vicariance or long-distance dispersal have been predominant (Kropf *et al.* 2006). A suitable species to investigate the role played by this geographical barrier is *C. ladanifer*, which is a perennial shrub displaying both unassisted diaspore and exclusive distribution in the Western Mediterranean region, from the Iberian Peninsula and southern France to northern Morocco and Algeria (Demoly & Montserrat 1993) (Fig. 1A).

Seed dispersal influences plant processes including maintenance of diversity and colonization of new land (Wang & Smith 2002). It is expected that lack of seed dispersal structures influences plant distribution, although occurrence of plants which display unassisted dispersal syndromes in remote territories has historically been intriguing (Carlquist 1967). In any case, it is exceedingly difficult to obtain accurate estimates of dispersal patterns of assisted or unassisted diaspore using direct observation because


A
B
C
40°N
10°W
5°W
0°
0 100 Km
Atlantic Ocean
Mediterranean Sea
Mediterranean Sea
5°W
35°N

long-distance dispersal events are often missed (Ouborg *et al.* 1999). Methods based on variation of genetic markers can otherwise detect the success of long-distance dispersal (i. e. colonization) and discriminate between alternative hypotheses for the origin of disjunct populations (Avise 2000). In the present study we analyse haplotype polymorphism to assess competing hypotheses on the origin of geographically disjunct populations and species of *Cistus*.

The plastid genome (cpDNA) is considered structurally stable, haploid, non-recombinant, generally uniparentally inherited (primarily maternally in angiosperms) and its variation structured geographically in a significant number of plant species (Soltis *et al.* 1997; Zhang *et al.* 2005). Accordingly, we examined plastid haplotype variation in *Cistus ladanifer* to: (1) analyze phylogeographical relationships among subspecies and populations; (2) infer patterns of historical dispersal and colonization in the Western Mediterranean; and (3) evaluate genetic consequences of physical barriers in isolation and differentiation after the reopening of the Strait of Gibraltar. We additionally estimated divergence times of *Cistus* lineages based on previous ITS and *trnK-matK* (Guzmán & Vargas 2005) regions and new *rbcL* sequences, as successfully analysed in angiosperm phylogenies (Wikström *et al.* 2001; Anderson *et al.* 2005; Bell & Donoghue 2005; Lavin *et al.* 2005; Linder *et al.* 2005; Magallón & Sanderson 2005). Molecular analysis combined with paleobotanical, ecological, and distributional data are lastly discussed to elucidate the colonization history of *Cistus* in the Mediterranean Basin.

←

Fig. 1. Distribution map (A) and geographical range of eight cpDNA haplotypes of *C. ladanifer* in the Iberian Peninsula and France (B) and North of Morocco (C). Numbers indicate haplotypes. Symbol "●" represents a population of *C. ladanifer* subsp. *africanus* population, "▲" of *C. ladanifer* subsp. *ladanifer* and "■" of *C. ladanifer* subsp. *sulcatus*.

2. Materials and methods

2.1. Study species

Cistus ladanifer is a self-incompatible and a predominantly entomophilous species (Talavera *et al.* 1993). Widely-open flower morphology and high quantity of pollen and nectar promote successful pollination by different groups of insects (over 100 species of beetles, bees and flies) (Herrera 1988; Bosch 1992). The fruits of *C. ladanifer* are globular, lignified capsules with 6-12 valves (Demoly & Montserrat 1993). Each fruit produces a large number of seeds (500-1000) with a great heterogeneity in germination success related to fire regimes (Thanos & Georghiou 1988; Valbuena *et al.* 1992). Seeds remain inside the capsules until summer, when fruit valves dehiscence and seeds fall near the mother plant (Bastida & Talavera 2002). *Cistus ladanifer* expands by seeds so, after an intense disturbance such a fire, the population is easily regenerated through plant and soil seed banks (Montgomery & Strid 1976; Arianoutsou & Margaris 1981; Valbuena *et al.* 1992).

Morphological variation of *Cistus ladanifer* is illustrated by key characters which differentiate the three subspecies (Demoly & Montserrat 1993): leaf shape and nerve type between subspp. *ladanifer* and *sulcatus*; and leaf base and petiole type between subspp. *ladanifer* and *africanus*. Although the distribution areas of the three subspecies occasionally overlap, subsp. *ladanifer* is primarily distributed in Iberian Peninsula, France and the northern Africa; subsp. *sulcatus* is endemic to SW Portugal (Algarve region); and subsp. *africanus* is scattered in southern Spain (Cádiz, Málaga) and more common in northern Africa. A phylogenetic hypothesis based on three nuclear and plastid markers shed light on the monophyly of *C. ladanifer*, but did not resolve subspecies relationships (Guzmán & Vargas 2005).

2.2. Sample strategy and DNA sequencing

A total of 56 individuals representing 48 different populations of *Cistus ladanifer* (one individual per population, in addition to three individuals from four Moroccan populations) were sampled (Table 1). A pilot study using only six *C. ladanifer*

Table 1. Cistaceae taxa sequenced for the *rcbL* gene and the *trnK-matK* and *trnS-trnG* spacers, plus ITS sequences from the GenBank. The majority of the DNA samples were used for sequencing the four regions. Dipterocarpaceae sequences were obtained from the GenBank. Material source, number of individuals per population[1], voucher reference, haplotype numbers and GenBank accession numbers are also indicated. Taxonomy follows that of Guzmán & Vargas (2005). Blotched (var. *maculatus*) and unblotched (var. *ladanifer*) individuals of *C. ladanifer* are indicated with solid (•) and open (○) circles after localities

Taxon	*Locality/source (number of individuals per population)*[1]	*Voucher*	*Haplotype Number*	*trnK-matK accession no.*	*trnS-trnG accession no.*	*ITS accession no.*	*rbcL accession no.*
Cistus L.							
Cistus albanicus E.F. Warb. ex Heywood	Cultivated	R. G. Page 8cBGA04 (MA)	-	DQ093010	-	DQ092964	Forthcoming
Cistus albidus L.	Spain, Madrid, Aldea del Fresno	P. Vargas 25PV03 (MA)	-	DQ092974	-	DQ092932	-
Cistus albidus L.	Morocco, Tetuán	P. Vargas 41PV03 (MA)	-	-	-	-	Forthcoming
Cistus chinamadensis Bañares et Romero	Canary Islands, La Gomera	R. G. Page 144BGA04 (MA)	-	DQ092987	-	DQ092943	-
Cistus chinamadensis Bañares et Romero	Canary Islands, La Gomera	Á. Fernández & J. Leralta 44BGA04 (MA)	-	-	-	-	Forthcoming
Cistus clusii Dunal	Spain, Málaga, Mijas	R. G. Page 8bBGA04 (MA)	-	DQ093009	-	DQ092963	Forthcoming
Cistus creticus L.	Greece, Olympus	P. Vargas 209PV04 (MA)	-	DQ092978	-	DQ092936	Forthcoming
Cistus crispus L.	Spain, Córdoba, Posadas	B. Guzmán 58BGA04 (MA)	-	DQ093013	-	DQ092967	Forthcoming
Cistus heterophyllus Desf.	Morocco, Beni-Hadifa	B. Guzmán 99BGA04 (MA)	-	DQ092989	-	-	Forthcoming
Cistus heterophyllus Desf.	Morocco	O. Filippi 7BGA04 (MA)	-	-	-	DQ092944	-
Cistus horrens Demoly	Canary Islands, Gran Canaria, San Bartolomé de Tirajana	B.Guzmán 5BGA05 (MA)	-	Forthcoming	-	-	Forthcoming
Cistus ladanifer L. ssp. *africanus* Dans.	Morocco, Asilah (3) ○	B. Guzmán 118BGA04 (MA)	4	Forthcoming	Forthcoming	-	-
"	Morocco, Beni-Hadifa ○	B. Guzmán 102BGA04 (MA)	4	Forthcoming	Forthcoming	-	-
"	Morocco, Grottes d'Hercules ○	P. Vargas 28PV03 (MA)	4	DQ093000	Forthcoming	DQ092955	-
"	Morocco, M'Diq	P. Vargas 45bPV03 (MA)	4[2]	Forthcoming	Forthcoming	-	-
"	Morocco, Taforalt ○	B. Guzmán 87BGA04 (MA)	7	Forthcoming	Forthcoming	-	-
"	Morocco, Targuist •	B. Guzmán 109BGA04 (MA)	4	DQ093001	Forthcoming	-	Forthcoming
"	Morocco, Tetuan (3) ○	P. Vargas 60PV03 (MA)	8	Forthcoming	Forthcoming	-	-
"	Morocco, Tleta-Ketama	V. Valcárcel 26VV03 (MA)	4	Forthcoming	Forthcoming	-	-
"	Morocco, Hejar Lesfar (3) ○	J. Martínez 121JM03 (MA)	5	Forthcoming	Forthcoming	-	-
"	Spain, Málaga, Sierra Bermeja ○	J. Martínez 253JM04 (MA)	6	Forthcoming	Forthcoming	-	-
Cistus ladanifer L. ssp. *ladanifer*	France, Saint Chinian	O. Filippi 17BGA05	1	Forthcoming	Forthcoming	-	-
"	Morocco, Bab-Taza •	B. Guzmán 115BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Morocco Chaouen (3) •	P. Vargas 46PV03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Morocco, Chaouen-Ketama	V. Valcárcel 20VV03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Morocco, Djebel Bouhalia	V. Valcárcel 32VV03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Aljezur	B. Guzmán 24BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Aljustrel ○	B. Guzmán 18BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Ourique ○	B. Guzmán 22BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Almería, Sierra de la Alhamilla ○	P. Vargas 179PV04 (MA)	6	DQ092997	Forthcoming	DQ092952	-
"	Spain, Almería, Torre Paraíso	P. Vargas 36PV05 (MA)	6	Forthcoming	Forthcoming	-	-
"	Spain, Cádiz, Almoraima	P. Vargas 14PV03 (MA)	6	Forthcoming	Forthcoming	-	-
"	Spain, Cáceres, Granadilla	B. Guzmán 3BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Ciudad Real, Sierra Madrona	B. Guzmán 6BGA06 (MA)	3	Forthcoming	Forthcoming	-	-
"	Spain, Córdoba, Posadas •	B. Guzmán 56BGA04 (MA)	2	Forthcoming	Forthcoming	-	-
"	Spain, Granada, Lanjarón	P. Vargas 124PV04 (MA)	6	Forthcoming	Forthcoming	-	-
"	Spain, Guadalajara, Hiendelaencina	B. Guzmán 26BGA03 (MA)	1	Forthcoming	Forthcoming	-	-

Table 1. *(Continued)*

"	Spain, Huelva, Hinojos o	E. Narbona 18EN03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Jaén, Bailén	J. Martínez 255JM04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, Boadilla del Monte o	B. Guzmán 7BGA03 (MA)	1	DQ092996	Forthcoming	-	Forthcoming
"	Spain, Madrid, Chapinería •	B. Guzmán 8BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, El Atazar o	B. Guzmán 28BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, El Escorial •	B. Guzmán 10BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, El Pardo	B. Guzmán 18BGA05 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, La Barranca	B. Guzmán 19BGA05 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, La Cabrera •	B. Guzmán 29BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Madrid, Manzanares El Real o	B. Guzmán 11BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Málaga, Casabermeja	P. Vargas 17PV06 (MA)	6	Forthcoming	Forthcoming	-	-
"	Spain, Orense, Laroca	J. Martínez 77BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Salamanca	P. Vargas 146PV05 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain Sevilla, Sierra Norte •	J. Martínez 258JM04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Toledo, Hinojosa de San Vicente	B. Guzmán 12BGA03 (MA)	1	Forthcoming	Forthcoming	-	-
"	Spain, Zamora	J. Martínez 272JM04 (MA)	1	Forthcoming	Forthcoming		-
Cistus ladanifer L. ssp. *sulcatus* (Demoly) P. Monts.	Portugal, Cabo San Vicente	B. Guzmán 36BGA04 (MA)	1	DQ092998	Forthcoming	DQ092953	-
"	Portugal, Cabo San Vicente	B. Guzmán 59BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Cabo Sardao	J. Arroyo 48BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Raposeira	B. Guzmán 38BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
"	Portugal, Sagres	B. Guzmán 29BGA04 (MA)	1	DQ092999	Forthcoming	-	Forthcoming
"	Portugal, Vila do Bispo	B. Guzmán 39BGA04 (MA)	1	Forthcoming	Forthcoming	-	-
Cistus laurifolius L.	Spain, Madrid, Las Rozas	P. Vargas 12PV03 (MA)	-	DQ093004	-	DQ092958	-
Cistus laurifolius L.	Spain, Jaén, Sierra de Segura	B. Guzmán 13BGA03 (MA)	-	-	-	-	Forthcoming
Cistus libanotis L.	Spain, Córdoba	R. G. Page 149BGA04 (MA)	-	DQ092993	-	DQ092948	Forthcoming
Cistus monspeliensis L.	Morocco, Grottes d'Hercules	P. Vargas 30PV03 (MA)	-	DQ093011	-	DQ092965	-
Cistus monspeliensis L.	Portugal, Sagres	B. Guzmán 35BGA04 (MA)	-	-	-	-	Forthcoming
Cistus munbyi Pomel	Morocco	O. Filippi 4BGA04 (MA)	-	DQ093006	-	DQ092960	Forthcoming
Cistus ochreatus C. Sm. ex Buch	Canary Islands, Gran Canaria	R. G. Page 150BGA04 (MA)	-	DQ092984	-	DQ092941	-
Cistus osbeckiifolius Webb ex Christ	Canary Islands, Tenerife	O. Filippi 160BGA04 (MA)	-	DQ092980	-	DQ092938	-
Cistus parviflorus Lam.	Greece, Crete	O. Filippi 6BGA04 (MA)	-	DQ092976	-	DQ092934	Forthcoming
Cistus populifolius L. ssp. *populifolius*	Spain, Ávila, Arenas de San Pedro	P. Vargas 5PV03 (MA)	-	DQ093003	-	-	-
Cistus populifolius L. ssp. *populifolius*	Morocco, Bab Taza	B. Guzmán 116BGA04 (MA)	-	-	-	-	Forthcoming
Cistus populifolius L. ssp. *major* (Dunal) Heywood	Portugal, Ourique	B. Guzmán 20BGA04 (MA)	-	-	-	DQ092957	-
Cistus pouzolzii Delile	Morocco, Ketama	S. L. Jury 698247MA	-	DQ093008	-	DQ092962	Forthcoming
Cistus psilosepalus Sweet	Spain, Ávila, Arenas de San Pedro	P. Vargas 7PV03 (MA)	-	DQ092994	-	DQ092949	Forthcoming
Cistus salviifolius L.	Spain, Ávila, Arenas de San Pedro	P. Vargas 6PV03 (MA)	-	DQ092990	-	DQ092945	Forthcoming
Cistus symphytifolius Lam.	Canary Islands, La Palma, La Cumbrecita	B. Guzmán 143BGA04 (MA)	-	DQ092983	-	DQ092940	Forthcoming
Crocanthemum Spach							
Crocanthemum chihuahuense S.Watson	Mexico, Michoacán	G. Calderón 527771MA	-	-	-	-	Forthcoming
Crocanthemum pringlei (S.Watson) Janch.	Mexico, Guanajuato	G. Calderón 527767MA	-	-	-	-	Forthcoming
Fumana (Dunal) Spach							
Fumana thymifolia (L.) Spach ex Webb	Portugal, Ferrerías	B. Guzmán 53BGA04 (MA)	-	DQ092968	-	DQ092926	-
Fumana ericoides	Spain, Almería, Cabo de Gata	B.Guzmán 3BGA06 (MA)	-	-	-	-	Forthcoming
Halimium (Dunal) Spach							
Halimium atriplicifolium (Lam.) Spach	Spain, Granada, Sierra Nevada	P. Vargas 120PV04	-	Forthcoming	-	-	-
Halimium atriplicifolium (Lam) Spach	Spain, Málaga, Coín	R. G. Page 155bBGA05 (MA)	-	-	-	-	Forthcoming
Halimium calycinum (L.) K. Koch	Portugal, Cabo Sardao	B. Guzmán 49BGA04 (MA)	-	-	-	DQ092931	-

Table 1. *(Continued)*

Halimium calycinum (L.) K. Koch	Portugal, Cabo de San Vicente	B. Guzmán 37BGA04 (MA)	-	-	-	-	**Forthcoming**
Halimium lasyocalycinum (Boiss. & Reut.) Gross ex Engl.	Morocco, Bab-Berred	P. Escobar 665/04 (MA)	-	-	-	-	**Forthcoming**
Halmium ocymoides (Lam.) Willk.	Portugal, Coimbra	R.G.Page 158BGA04 (MA)	-	**Forthcoming**	-	-	-
Halimium ocymoides (Lam.) Willk.	Spain	R. G. Page 158bBGA04 (MA)	-	-	-	-	**Forthcoming**
Halimium umbellatum (L.) Spach	Spain, Madrid, Tres Cantos	P. Vargas 71BGA04 (MA)	-	DQ092972	-	DQ092930	-
Helianthemum Mill.							
Helianthemum scopulicolum L.	Cultivated	B. Guzmán 67BGA04 (MA)	-	DQ092970	-	DQ092928	**Forthcoming**
Helianthemum squamatum (L.) Dum. Cours.	Cultivated	B. Guzmán 70BGA04 (MA)	-	DQ092969	-	DQ092927	**Forthcoming**
Tuberaria Dunal							-
Tuberaria guttata (L.) Fourr.	Portugal, Vila do Bispo	B. Guzmán 44BGA04 (MA)	-	DQ092971	-	DQ092929	**Forthcoming**
Tuberaria globulariifolia (Lam.) Gallego	Spain, Orense, Sierra de Xures	J. Martínez 269JM04 (MA)	-	**Forthcoming**	-	-	
Dipterocarpaceae							
Dipterocarpus C.F.Gaertn.							
Dipterocarpus glandulosus Thw.	Sri Lanka, Kanneliya Forest Reserve	Gamage *et al.* (2003, 2006)	-	AB246477	-	-	-
Hopea Roxb.							
Hopea hainanensis Merr. & Chun	-	Cho *et al.* (unpublished data)	-	-	-	-	AJ247623.1
Hopea wightiana Wall.	Malayasia, Frim Arboretum	Gamage *et al.* (2003, 2006)	-	AB246461	-	-	-
Shorea Roxb. ex C.F.Gaertn							
Shorea affinis (Thwaites) P.S.Ashton	Sri Lanka, Kottawa Arboretum	Gamage *et al.* (2003, 2006)	-	AB246471	-	-	-
Shorea talura Roxb.	-	Yuan *et al.* (unpublished data)	-	-	-	-	AY328198.1

[1] One individual per population except in four *Cistus ladanifer* populations in which three individuals were sequenced.

[2] Haplotype 9 when coding indels

populations was initially performed to search for the most variable plastid sequences (*trnL-trnF, trnK-matK, trnS-trnG, rbcL*). Standard primers were used for amplification of the *trnK-matK* spacer (*trnK*-3914F, *matK*-1470R) (Johnson & Soltis 1994), the *trnL* (UAA)-*trnF* (GAA) spacer (Taberlet *et al.* 1991) and the *trnS* (GCU)-*trnG* (UCC) spacer (Hamilton 1999). The *rbcL* exon was amplified in two overlapping segments using the following combination of primers: 1F-724R and 636F-1460R (Savolainen *et al.* 2000). As *trnK-matK* and *trnS-trnG* sequences displayed the highest variation at the species level, sequencing of *trnL-trnF* and *rbcL* regions was discarded to reconstruct phylogeographic patterns of *C. ladanifer*. In addition, a data set of *trnS-G* and *trnK-matK* sequences of *C. ladanifer* (one per haplotype) plus the other 10 white-flowered species, which form a well-supported clade (Guzmán & Vargas 2005), and two purple-flowered species used as the outgroup, was analyzed to investigate monophyly and haplotype ancestry of *C. ladanifer* (Table 1). Three data sets (*rbcL, trnK-matK,* ITS) were additionally used to estimate divergence times of *Cistus* and related lineages. To perform a comparative study, the majority of the samples used in the *trnK-matK* and ITS sequencing study (Guzmán & Vargas 2005) were employed to sequence *rbc*L (Table 1). Dipterocarpaceae sequences of *matK* and *rbcL*, as retrieved from the Genbank, were used in the different analyses as outgroup sequences (Table 1).

Procedures used for DNA sequencing are given in Guzmán & Vargas (2005), except for specific amplifications. After 1-3 min pretreatment at 94 °C, PCR conditions for *rbcL, trnK-matK* and *trnS-trnG* amplification were: 24-28 cycles of 1 min at 94 °C, 30 s-1 min at 48-50-55 °C and 1-2 min at 72 °C.

Maternal inheritance of plastid DNA in *Cistus* species was assessed by analysis of *trnK-matK* and *trnS-G* sequences in controlled hybrids: *C. parviflorus* x *C. laurifolius, C. libanotis* x *C. ladanifer* (Beatriz Guzmán, Robert G. Page, Pablo Vargas, unpublished data).

2.3. Haplotype data analysis

Cistus ladanifer sequences of *trnK-matK* and *trnS-trnG* were combined and aligned by hand given the low number of gaps across sequences. Relationships among the haplotypes were inferred using the software TCS 1.21 (Clement *et al.* 2000), a method of statistical parsimony to construct haplotype phylogenetic networks (Templeton *et al.* 1992). The maximum number of differences among haplotypes, as a result of single substitutions, was calculated by treating gaps as missing data. Additionally, we analysed a matrix by recoding indels as new characters, but removing the mononucleotide repeat stretches (poli-T and poli-A) because homology is highly uncertain for this polymorphism type (Kelchner 2000). In particular, we employed Simmons and Ochotorena's (2000) "simple indel coding" approach. Maximum Parsimony (MP) analyses were performed using PAUP (Swofford 2002). The parameters for the heuristic search were: 100 replicates, random taxon-addition sequences, tree-bisection-reconnection (TBR) branch swapping and the options Multrees and Steepest in effect. Robustness of clades was estimated using 1,000,000 bootstrap replicates (fast stepwise-addition, Mort *et al.* 2000). Bayesian Inference (BI) was inferred using two identical searches with ten million generations each (chain temperature=0.2; sample frequency=100). In both runs probabilities converged on the same stable value approximately after generation 40,000. A 50% majority–rule consensus tree was calculated using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro *et al.* 2003).

2.4. Estimating lineages divergences

Tree topology and branch lengths from *rbcL, trnK-matK* and ITS sequences of the Cistaceae were obtained using Maximum Likelihood (ML), as performed in PAUP (Swofford 2002). Maximum Likelihood (ML) and Maximum Parsimony (MP) bootstrap searches were conducted using a fast stepwise-addition (1,000,000 replicates, Mort *et al.* 2000). MrModeltest 1.1b (Posada & Crandall 1998; Nylander 2002) was employed to determine the best model of sequence evolution. To estimate divergence times we

employed the most similar ML tree to the MP consensus tree based on the three regions presented in Guzmán and Vargas (2005).

To check the constancy of substitution rates we used the Langley and Fitch (LF) test (Magallón & Sanderson 2005). We rejected the null hypothesis of constant rate and, then, used the penalized likelihood smoothing as implemented in r8s (PL, Sanderson 2002). PL was implemented with the Truncated Newton (TN) algorithm and the following parameters: collapse; num_time_guesses=5 and num_restarts=5. As recommended in r8s manual, we pruned the extraoutgroup (*Hopea hainanensis* in *rbcL* data set, *Dipterocarpus glandulosus* in *trnK-matK* data set and *Fumana thymifolia* in ITS data set). The smoothing parameter for PL method was calculated by a cross-validation procedure with the following parameters: cvstart=0.5; cvinc=0.5; cvnum=10. Crossvalidation suggested that the best smoothing parameter was 3.2 for *rbcL*, 3.2 for *trnK-matK* and 1000 for ITS data sets. To convert relative divergence times into absolute time units we used calibration points. There are three sources of independent chronological information: the fossil record, geological information and molecular-based ages estimates (Magallón 2004). Nodes can be constrained with a minimum age (i.e., estimated ages are not allowed to be younger than the constraint) and/or with a maximum age (i.e., estimated ages are not allowed to be older than the constraint). Nodes can also be fixed at a value, but ages of fixed nodes are not longer estimated so no node was fixed for the analyses. Fossil data are poor in the Cistaceae and most of them are pollen records. We used only the most reliable dates of pollen records and macrofossils to constrain some nodes with a maximum age. Palynological studies identified *Helianthemum* pollen in Upper Miocene formations (11 Ma) from France (Naud & Suc 1975) and *Tuberaria* pollen in Pliocene formations (5.3 Ma) from Germany (Menke 1976). Additionally, a macrofossil of a reproductive structure from Germany, described in Palibin (1909) as *Cistinocarpum roemeri* Conw., was used to constrain the split of the Cistaceae with a maximum age of 28 Ma (Middle Oligocene). A basal point was used to calibrate the crown group of the Cistaceae. We used the split age between the Dipterocarpaceae and the Cistaceae obtained by Wikström *et al.* (2001), so we

constrained the node with a minimum age of 23 Ma and a maximum age of 39 Ma. Given insufficient sequence similarity or alignability between Cistaceae and Dipterocarpaceae samples, we did not include Dipterocarpaceae sequences to perform this basal constraint in the ITS analysis.

3. Results

3.1. *Analysis of* Cistus ladanifer *haplotypes*

Sequences length was 1309-1314 bp for *trnK-matK* and 633-646 bp for *trnS-trnG* (Table 2). The same plastid sequences were detected in three individuals of four populations sampled in a Moroccan transect from an area where numerous haplotypes were found (Table 1). The combined data of *trnS-trnG* and *trnK-matK* sequences (112) distinguished

Table 2 List of haplotypes found in 48 *Cistus ladanifer* populations. Variable sites (excluding mononucleotide repeat stretches) of the sequences of the two plastid DNA fragments (*trnK-matK*, *trnS-trnG*) are shown. Nucleotide position in each data set is numbered from the 5′ to the 3′ DNA ends

Nucleotide	*trnK-matK*					*trnS-trnG*								
position	155	162	964	1022	1046	91	118	321	331	384	461	470	498	501
Haplotype														
1	T	-	G	G	T	G	C	A	A	G	T	C	†	*
2	T	-	G	G	T	G	C	A	C	G	T	C	†	*
3	T	-	G	G	T	G	C	T	C	G	T	C	†	*
4	T	-	G	G	C	G	T	A	A	T	T	A	†	-
5	T	-	G	G	C	G	T	A	A	T	C	A	†	-
6	T	-	A	G	C	G	T	A	A	T	T	A	†	-
7	T	-	G	T	C	G	T	A	A	T	T	A	†	-
8	A	‡	G	G	C	T	T	A	A	T	T	A	-	-
9	T	-	G	G	C	G	T	A	A	T	T	A	-	-

‡ GAATT
† AAA
* CAAAACTAAA

eight nucleotide haplotypes (Table 1), which are distributed in the Iberian Peninsula and France (4 haplotypes) and northern Africa (5 haplotypes). Only haplotype 1 was found in the two geographical areas (31 European and African populations, i.e. 65% of all populations). In Morocco, haplotype 1 was only present in subsp. *ladanifer* populations, whereas in the Iberian Peninsula this haplotype was shared by subspp.

ladanifer and *sulcatus* (Fig. 1B). Haplotype 4 was distributed in six African populations (12.5% of all populations) of subsp. *africanus* from Morocco (Table 1 and Fig. 1C). In contrast, the Andalusian population of subsp. *africanus* displayed the same haplotype (6) as the five populations sequenced of subsp. *ladanifer* from Andalusia. The five remaining haplotypes (2, 3, 5, 7, 8) were found exclusively in a single population each. Accordingly, only two haplotypes were shared between subspecies (haplotype 1 in subspp. *ladanifer, sulcatus;* haplotype 6 in subspp. *ladanifer, africanus*) (Table 1).

TCS constructed a single network of the eight *C. ladanifer* haplotypes connected through haplotype 5 to those of the other ten white-flowered species (Fig. 2A). In the *C. ladanifer* clade, two groups of haplotypes (1-2-3/4-5-6-7-8) were separated by three missing (extinct or not found) haplotypes. This split into two groups of haplotypes is not fully related to two geographical areas (Iberian Peninsula-France and Morocco). Occurrence of the interior haplotype 1 in both continents links a high number of populations and distributional areas. The network depicted the interior haplotype 4 as the one with more mutational connections (five connections). A single mutation in contrast connects the tip haplotype 6 from Iberia with the primarily African group of haplotypes indicating disassociation between population distributions and the haplotype genealogy. When coding indels (one within the partial *trnK-matK* sequences and two within the *trnS-G* spacer sequences; Table 2) TCS constructed a network with nine haplotypes (results not shown). A new haplotype (9) was found in Morocco (one population from M'Diq), which is connected by one mutational step to haplotype 4 and by two mutational steps to haplotype 8. The two haplotype clades of plants primarily distributed in Western Europe and northern Africa are also retrieved and major tip and interior haplotype relationships. We hereafter considered the network analysis with no indel coding for the shake of brevity.

Artificial crossings generated two controlled hybrids for reconstruction of haplotype inheritance. In all cases, sequence polymorphisms of the individuals acting as female plants were inherited by F1 individuals.

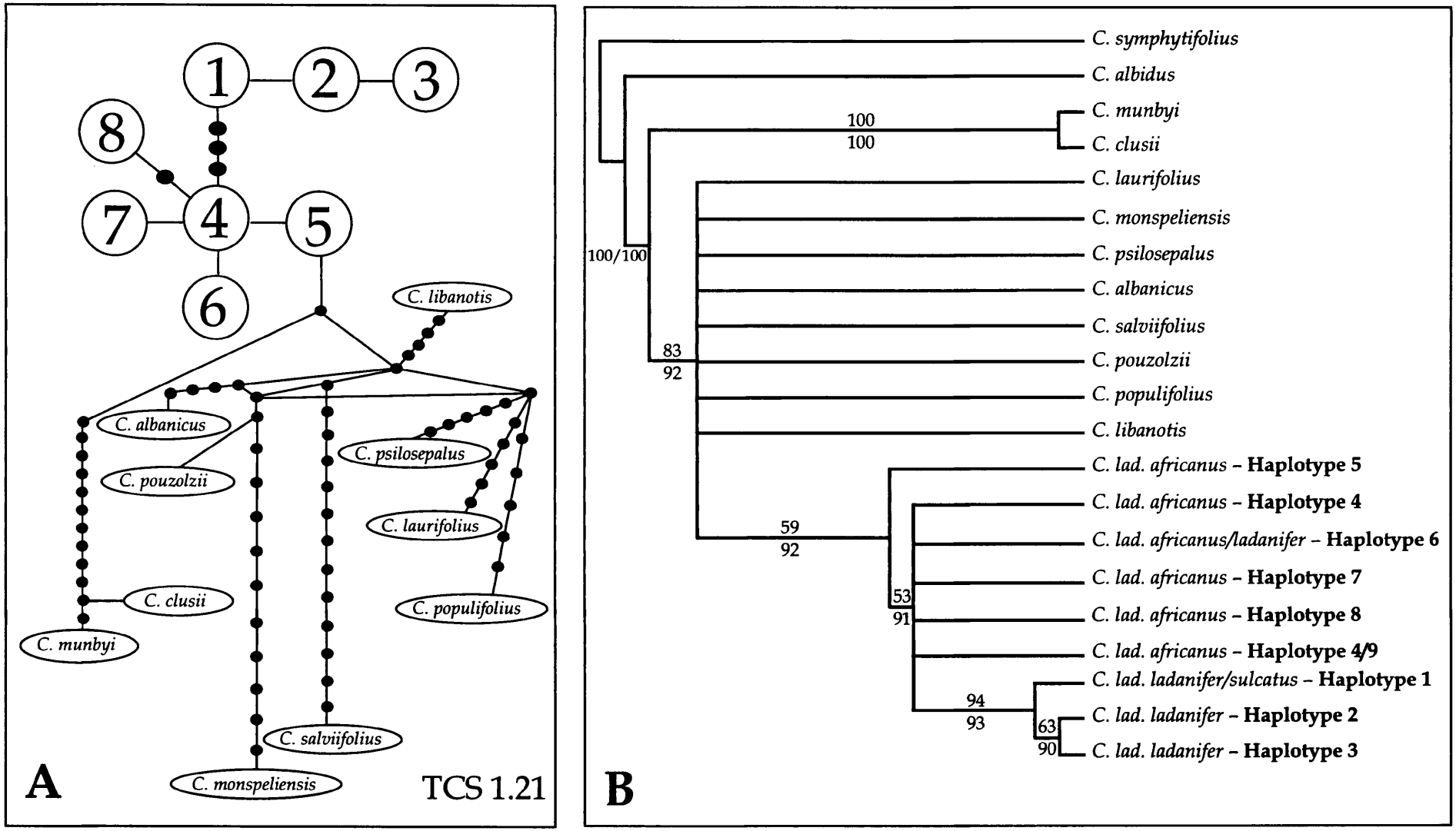


Fig. 2 Statistical parsimony network based on *trnS-trnG* and *trnK-matK* sequences of *C. ladanifer* and white-flowered related species (a). *Cistus ladanifer* haplotypes are indicated by the numbers (1 to 8), lines indicate a single nucleotide substitution, and "•" represents missing haplotypes (extinct or not found). Strict consensus tree (b) of 9493 shortest trees of 157steps (CI=0.94; RI=0.90; RC=0.85) from the combined analysis of *trnS-G* and *trnK-matK* sequences. Numbers above branches are bootstrap values. Numbers below branches show Bayesian posterior probabilities.

3.2. Phylogenetic analysis

The aligned length of the combined *trnK-matK* and *trnS-G* sequences of the 11 white-flowered species of *Cistus* (including the nine haplotypes of *C. ladanifer*) was 2106 bp and included 11 coded indels. Fifty-two of the 129 variable characters were phylogenetically informative. MP analysis generated 9493 trees of 157 steps with a consistency index (CI) of 0.94, a retention index (RI) of 0.90, and a rescaled consistency index (RC) of 0.85 (Fig. 2B). A Bayesian tree was reconstructed using GTR+G, which was retrieved as the simplest model of sequence evolution, and was mostly consistent with the strict consensus tree of the MP trees (Fig. 2B). The MP tree recognizes *C. ladanifer* as a monophyletic group with 59% bootstrap value (BS) and 92% posterior probability (PP) and haplotypes 1, 2 and 3 as a well-resolved clade (94% BS, 93% PP). Bayesian analysis retrieved haplotype 5 (Hejar Lesfar, Morocco) as sister (91% PP) to the other haplotypes, as the MP analysis did, although with weak support (53% BS). This result fully agrees with the network analysis (see above).

3.3. rbcL *sequence variation in the Cistaceae*

The length of *rbcL* sequences ranged from 1459 bp (*Helianthemum squamatum*) to 1438 bp (11 *Cistus* plus two *Halimium* species). The aligned matrix contained 1459 characters, of which 167 were variable and 121 parsimony informative. Within the Cistaceae, 94 characters were variable and 83 parsimony informative; and within *Cistus*, 25 characters were variable and 23 parsimony informative.

3.4. Divergence times

Although the assumption of the hypothesis of equivalent rates of sequence evolution across lineages was rejected, estimates of divergence times using penalized likelihood were mostly congruent between the three data sets (ITS, *rbcL*, *trnK-matK*) (Table 3; Fig. 3). Our results indicate that after the divergence between the Dipterocarpaceae and the Cistaceae (Wikström *et al.* 2001) differentiation of the Cistaceae genera took place during a long period of time (Miocene). All the analyses are congruent with a

Table 3. Settings and results of the Maximum Likelihood analyses for the three DNA fragments

	Model	Base frequencies	Gamma distribution	Best Score (-lnL)	No. trees
*rbc*L	GTR+G	A = 0.279; C = 0.187; G = 0.244; T = 0.289	0.144	3539.337	24
trnK-matK	GTR+G	A = 0.321; C = 0.156; G = 0.166; T = 0.356	1.141	4562.354	6
ITS	GTR+I+G	A = 0.180; C = 0.343; G = 0.301; T = 0.168	0.144	2730.673	20

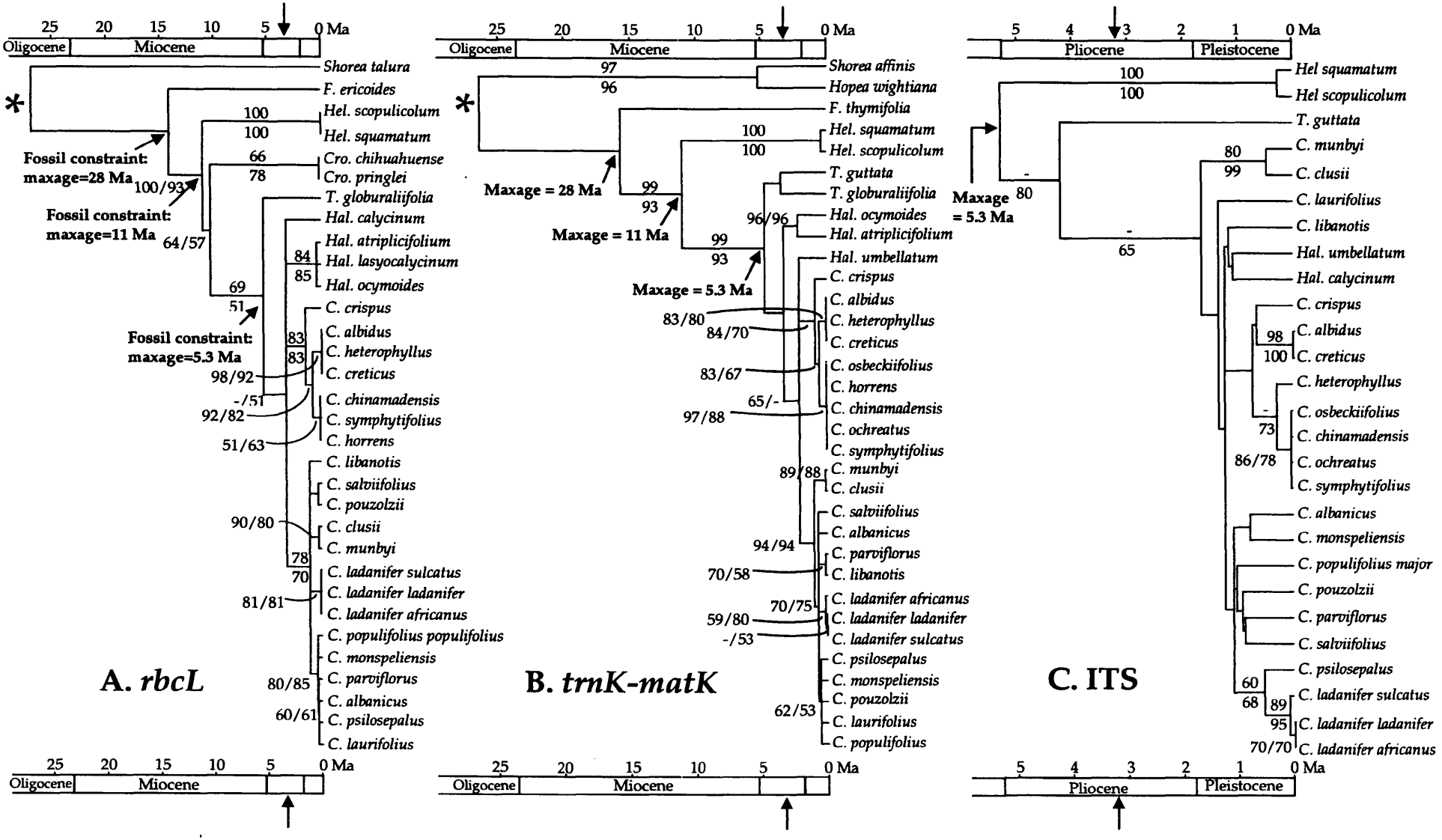


Fig. 3. Chronograms of maximum likelihood trees based on *rbcL* (a), *trnK-matK* (b) and ITS (c) sequences. Branch lengths represent million of years (Ma). Values above and bellow branches are bootstrap values of the ML and MP analyses, respectively. Three fossils and one estimated date (represented with an asterisk) of the Cistaceae divergence (Wikström *et al.* 2001) were used to implement the analyses. The onset of the Mediterranean climate (c. 3.2 Ma) is indicated by an arrow on the time scale.

differentiation of the complex *Cistus-Halimium* primarily in the Pliocene (crown age: 3.88 Ma, *rbcL*; 3.26 Ma, *trnK-matK*; 1.66 Ma, ITS), followed by divergence of *Cistus* species in two major groups: one lineage of purple-flowered species (*C. crispus, C. albidus, C. heterophyllus, C. creticus, C. chinamadensis, C. symphytifolius, C. horrens, C. ochreatus,* and *C. osbeckiifolius*) and other lineage of white-flowered species plus the pinkish-flowered *Cistus parviflorus*. Species divergence took simultaneously place in the purple- and white-flowered groups (1.80 Ma and 1.43 Ma in the analysis *rbcL*; 0.88 Ma and 1.01 Ma in *trnK-matK*; 0.73 Ma and 1.09 Ma in ITS; respectively). All these divergence times postdate the re-opening of the Strait of Gibraltar (c. 5 Ma), suggesting that disjunct distributions of *Cistus* species/lineages across this sea barrier are due to dispersal rather than vicariance.

4. Discussion

4.1. Haplotype distribution across the Strait of Gibraltar

The Strait of Gibraltar displays a high number of shared species as a result of historical connections between Western Europe and Africa. Hierarchic levels of lineage diversity in the Western Mediterranean reveal that species diversity (alpha diversity) parallels that of lineage diversity in *Cistus* (Guzmán & Vargas 2005). Haplotype richness of *C. ladanifer* in the hotspot of the Strait of Gibraltar agrees with a scaled diversity at the population, subspecies and species levels. Within this hotspot, a specific pattern of haplotype richness in certain areas (five haplotypes in the southern side vs. four in the northern side) differs depending on the plant group: one in N Africa and four in Iberia for *Hedera* spp. (Grivet & Petit 2002); three in N Africa and nine in Iberia for *Quercus ilex* (Lumaret *et al.* 2002); two in N Africa and six in Iberia for *Frangula alnus* (Hampe *et al.* 2003); two in N Africa and two in Iberia for *Quercus suber* (Lumaret *et al.* 2005); three in N Africa and three in Iberia for *Saxifraga globulifera* (P. Vargas, unpublished).

Recent studies, however, concluded that taxon (morphological) richness is not always a good surrogate for phylogenetic diversity in other Mediterranean floristic regions (Forest *et al.* 2007). The two sides of the Strait of Gibraltar also harbour high

levels of morphological diversity, as reflected by the occurrence of two (northern Morocco) and three (southern Iberia) subspecies and two varieties of subsp. *ladanifer* in both continents. Taxonomic varieties of subsp. *ladanifer* are not correlated with particular haplotypes since the individuals sequenced with (var. *maculatus*) and without (var. *ladanifer*) blotched petals share the same haplotype and are intermingled in numerous populations from northern Africa and southern Iberia (Table 1) (but see chemotaxonomic results in Robles *et al.* 2003). In contrast, subspecies delimitation appears mostly concordant with haplotype distribution. Population differentiation of subspecies reveals that subsp. *ladanifer* contains a lower number of haplotypes (1, 2, 3, 6) in 32 populations than subsp. *africanus* (4, 5, 6, 7, 8) in 10 populations, while the restricted southern Portuguese subsp. *sulcatus* displays a single haplotype (1) (Table 1). Six of the eight plastid haplotypes are exclusive to particular subspecies. A higher haplotype exclusiveness is observed in subsp. *africanus* (haplotypes 4, 5, 7, 8), whereas subsp. *ladanifer* shows only two private haplotypes (2, 3) and subsp. *sulcatus* none. Further phylogeographical analysis of a larger number of plant groups are needed to determine whether subspecies differentiation mostly reflects haplotype diversity in the Western Mediterranean.

4.2. Phylogeographical patterns and intercontinental colonizations of Cistus ladanifer

Two cpDNA lineages were identified across the distribution area of *Cistus ladanifer* (Fig. 2): one primarily European (including haplotypes 1, 2, 3) and other primarily African (including haplotypes 4, 5, 6, 7, 8). Reconstructions of geographical ancestry of haplotypes point out a major centre of diversity in northern Africa. The *C. ladanifer* clade is connected through northern African haplotype 5 to the haplotypes of the other ten white-flowered species (Fig. 2A). The same result is obtained in the phylogenetic reconstruction, where haplotype 5 is sister to the other seven haplotypes of *C. ladanifer* (Fig. 2B).

Considering origin of *C. ladanifer* in northern Africa, population disjunctions may be the result of colonizations from the southern side of the Strait of Gibraltar. Results of phylogenetic and network analyses fit into a diversification pattern in which a northern African ancestor spawned at least three new lines of evolution in northern Africa (haplotypes 1/7/8). Both the presence of the haplotype 1 in both continents and the haplotype relationships in the network analysis suggest a first colonization of migrants with this haplotype followed by differentiation (haplotypes 2-3) in the Iberian Peninsula. An early colonization of haplotype 1 is consistent with the molecular-clock estimate (see below *The colonization history of* Cistus). A different line of evolution (haplotype 6) closely related to the African lineage (a single mutational step from haplotype 4) was found exclusively in southern Iberia (Fig. 2A). The number of plastid mutations (4) is so high to reconcile the tip haplotype 6 and the primarily European lineage that we infer the most congruent hypothesis: relatively recent long-distance dispersal resulting in a new colonization of southern Iberia. This result contrasts with previous phylogeographical analyses of Mediterranean plant groups also displaying unassisted dispersal syndromes and past genetic fragmentation (Vargas *et al.* 1999; Bittkau & Comes 2005).

Factors affecting colonization success of plant groups include pollen and seed dispersal, seed germinability, habitat preferences, plant-growth conditions, breeding system, plant-animal interactions, among others (Wang & Smith 2002). Assignation of paternal vs. maternal inheritance of cytoplasmic organelles is crucial to infer pollen vs. seed routes of dispersal. Our haplotype network is consistent with plastid reconstruction of seed dispersal, given that controlled hybrids inherited plastid sequences from individuals acting as female plants. Irrespective of limited dispersal abilities in *Cistus*, a wider window of opportunity for successful colonization of xerophilous organisms appears to have occurred on both sides of the Strait of Gibraltar since establishment of the Mediterranean climate (F. Rodríguez *et al.*, unpublished). Absence of both dispersal mechanisms and extended habitats appears to have been decisive to unsuccessful colonization. In fact, it has been documented that the Strait of

Gibraltar have played a major geographical barrier for plants with both small seeds in dehiscent capsules and particular habitat needs such as *Saxifraga globulifera* (Vargas *et al.* 1999) and *Androcymbium gramineum* (Caujapé-Castells & Jansen 2003), but not for others displaying specific dispersal mechanisms such as *Frangula alnus* (Hampe *et al.* 2003), *Quercus ilex* (Petit *et al.* 2005), *Quercus suber* (Lumaret *et al.* 2005), and *Hypochaeris salzmanniana* (Ortiz *et al.* 2007).

Occurrence of *C. ladanifer* in successional stages following woodland disturbances of *Quercus suber* (Lumaret *et al.* 2005) and *Quercus ilex* (Petit *et al.* 2005), with which not only shares similar distributions but also a phylogeographical patterns of migration across the Strait of Gibraltar, indicates a shared history of colonization at some extent (Fauquette *et al.* 1999). Suitable ecological conditions after *Q.suber/Q. ilex* woodland disturbance are invoke to account for successful intercontinental colonization of *C. ladanifer* irrespective of limited dispersal abilities.

4.3. Ecology and population expansion of Cistus ladanifer

Cistus ladanifer has a high reproductive potential to colonize and form part of the Mediterranean scrub, particularly after fire (Corral *et al.* 1990; Thanos *et al.* 1992; Pérez-García 1997). This species displays high fruit and seed sets (65-90%, Talavera *et al.* 1993, B. Guzmán et al., unpublished data), compared with other hermaphrodite and self-incompatible plant species (Sutherland & Delph 1984). Because of the high number of flowers per plant (more than 500, in some cases) and the high number of ovules per flower (more than 1000), an individual can release thousands of seeds per year from its dehiscent capsules. Massive seed dispersal by gravity (barochory), high rates of germinability and successful range expansion in Mediterranean environments may account for rapid colonization of *C. ladanifer* after woodland disturbance (Luis-Calabuig *et al.* 2000).

Additionally to limited pollen dispersal by insects (Herrera 1988; Bosch 1992), poor seed dispersal capacity is expected to agree with the observed phylogeographical patterns in *C. ladanifer*. Dispersal of 26% of seeds occurs only at 20-60 cm from the

canopy edges of its mother plant (Bastida & Talavera 2002). Once in the soil, seeds of *C. ladanifer* can undergo a secondary dispersal by granivorous ants, which could benefit seedlings survival by eliminating adult plants influence by a few meters. Homogeneous distribution of haplotype 1 (haplotypes 2 and 3 only found in two populations each) in Europe may be the result of a gradual expansion following a leading edge model when recovering Mediterranean habitats after glaciations. Although glaciation survival of *C. ladanifer* plants containing the ancestral haplotype 1 in northern Iberia and southern France is also congruent with our genealogical reconstruction, historical conditions do not support this alternative hypothesis. Range expansion from southern Iberian refugia is, in contrast, suggested by circumstantial evidence given that northern areas were climatically uninhabitable for strict Mediterranean species, such as *C. ladanifer*, across the harsh episodes of the last glaciations (Hewitt 2000). Pollen records attributed to *C. ladanifer* support a long-stand presence in southern Iberia, but a discontinuous occurrence northwards (Pons & Reille 1988). It has been documented that Mediterranean species were restricted to refugia of southern Iberia, followed by range expansion in post-glacial periods (Hampe *et al.* 2003; Valcárcel *et al.* 2003; Vargas 2003). This phylogeographical structure (high genetic variability in the refuge area and genetic uniformity in the recolonized areas) resulted in a single haplotype also found across populations of European ivies (Grivet & Petit 2002; Valcárcel *et al.* 2003). Patchy distribution of haplotypes in the highly diverse northern Africa (Fig. 1C) is, in contrast, congruent with a long evolutionary history in a hotspot (see above).

4.4. The colonization history of Cistus

The Dipterocarpaceae and the Cistaceae split during the Eocene-Oligocene (Wikström *et al.* 2001), when a subtropical climate existed. The three chronograms (Fig. 3) are congruent with a divergence pattern of three Cistaceae genera (*Tuberaria, Halimium, Cistus*) at the Miocene-Pliocene boundary (Messinian, 5.96-5.33 Ma; Duggen *et al.* 2003), when a xeric climate was predominant (Fauquette *et al.* 1999). In particular, the differentiation of the complex *Cistus-Halimium* appear to have occurred at most in the Pliocene (crown age: 3.88 Ma, *rbcL;* 3.26 Ma, *trnK-matK;* 1.66 Ma, ITS). Accordingly,

lineage divergence within both the purple-flowered and white-flowered *Cistus* groups did not predate the Messinian, which coincide with the re-opening of the Strait of Gibraltar (5.3 Ma, Krijgsman *et al.* 1999). Given that extant *Cistus* species were not in existence before this geologic event, post-Mesisinian sea expansion and subsequent land fragmentation imply multiple events of colonization by long-distance dispersal. In particular, lineage radiation of *Cistus* appears to be closely related to the onset of the Mediterranean climate about 3.2 Ma (Suc *et al.* 1995) (Fig. 3). We interpret continuous colonization, speciation and recolonization processes in the history of *Cistus*, which was clearly favoured by the recurrent expansion of xeric habitats after establishment of the Mediterranean climate.

Further support for the long-distance hypothesis and the colonization potential of *Cistus* is found in Macaronesian plants. Although the five endemic, purple-flowered species distributed in five islands are the result of a single introduction to the Canary Islands (Guzmán & Vargas 2005), occurrence of the white-flowered *C. monspeliensis* in Macaronesia indicates multiple dispersal to two oceanic archipelagos separated at least 100 km (Lanzarote, Canary Islands) and 900 km (Madeira) from the continent (Hansen & Sundig 1993). Absence of specific structures did not necessarily impede neither medium (interisland) nor long-distance dispersal. In fact, a phylogeny-based survey of plant syndromes of the Canarian flora reveals the success of 26% of ancestors with unassisted diaspore for long-distance dispersal (Vargas 2007). Again, Mediterranean habitat conditions in Macaronesia may have been crucial for *Cistus* establishment on the Canary Islands.

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References

Albaladejo RG, Fuertes J, Aparicio A, Nieto G (2005) Contrasting nuclear-plastidial phylogenetic patterns in the recently diverged Iberian *Phlomis crinita* and *P. lychnitis* lineages (Lamiaceae). *Taxon* **54**, 987-998.

Alfaro ME, Zoller S, Lutzoni F (2003) Bayes or bootstrap? a simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution* **20**, 255-266.

Anderson CL, Bremer K, Friis EM (2005) Dating phylogenetically basal eudicots using *rbc*L sequences and multiple fossil reference points. *American journal of botany* **92**, 1737-1748.

Arianoutsou M, Margaris NS (1981) Early stages of regeneration after fire in a phryganic ecosystem (east Mediterranean). I. Regeneration by seed germination. *Biologie et écologie méditerranéenne* **8**, 119-128.

Avise JC (2000) *Phylogeography: the history and formation of species* Harvard University Press, Cambridge.

Bastida F, Talavera S (2002) Temporal and spatial patterns of seed dispersal in two *Cistus* species (Cistaceae). *Annals of Botany* **89**, 427-434.

Bell CD, Donoghue MJ (2005) Dating the Dipsacales: comparing models, genes, and evolutionary implications. *American Journal of Botany* **92**, 284-296.

Bittkau C, Comes HP (2005) Evolutionary processes in a continental island system: molecular phylogeography of the Aegean *Nigella arvensis* alliance (Ranunculaceae) inferred from chloroplast DNA. *Molecular Ecology* **14**, 4065-4083.

Blondel J, Aronson J (1999) *Biology and wildlife of the Mediterranean region* Oxford University Press, Oxford.

Bosch J (1992) Floral biology and pollinators of three co-ocurring *Cistus* species (Cistaceae). *Botanical Journal of the Linnean Society* **109**, 39-55.

Carlquist S (1967) The biota of long-distance dispersal. V. Plant dispersal to Pacific Islands. *Bulletin of the Torrey Botanical Club* **94**, 129-162.

Caujapé-Castells J, Jansen RK (2003) The influence of the Miocene Mediterranean desiccation on the geographical expansion and genetic variation of *Androcymbium gramineum* (Cav.) McBride (Colchicaceae). *Molecular Ecology* **12**, 1515-1525.

Caujapé-Castells J, Jansen RK, Membrives N, *et al.* (2001) Historical biogeography of *Androcybium* Willd. (Colchicaceae) in Africa: evidence from cpDNA RFLPs. *Botanical Journal of the Linnean Society* **136**, 379-392.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Coleman M, Liston A, Kadereit JW, Abbott RJ (2003) Repeat intercontinental dispersal and Pleistocene speciation in disjunct Mediterranean and desert *Senecio* (Asteraceae). *American Journal of Botany* **90**, 1446-1454.

Corral R, Pita JM, Pérez-García F (1990) Some aspects of seed germination in four species of *Cistus* L. *Seed Science and Technology* **18**, 321-325.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Duggen S, Hoernle K, van den Bogaard P, Rüpke L, Morgan JP (2003) Deep roots on the Messinian salinity crisis. *Nature* **422**, 602-606.

Fauquette S, Suc JP, Guiot J, *et al.* (1999) Climate and biomes in the West Mediterranean area during the Pliocene. *Palaeogeography, Palaeoclimatology, Palaeoecology* **152**, 15-36.

Forest F, Grenyer R, Rouget M, *et al.* (2007) Preserving the evolutionary potential of floras in biodiversity hotspots. *Nature* **445**, 757-760.

Gamage DT, de Silva MP, Inomata N, Yamazaki T, Szmidt AE (2006) Comprehensive molecular phylogeny of the sub-family Dipterocarpoideae (Dipterocarpaceae) based on chloroplast DNA sequences. *Genes and genetic system* **81**, 1-2.

Gamage DT, de Silva MP, Yoshida A, Szmidt AE, Yamazaki T (2003) Molecular phylogeny of Sri Lankan Dipterocarpaceae in relation to other Asian Dipterocarpaceae based on chloroplast DNA sequences. *Tropics* **13**, 79-87.

Grivet D, Petit RJ (2002) Phylogeography of the common ivy (*Hedera* sp.) in Europe: genetic differentiation through space and time. *Molecular Ecology* **11**, 1351-1362.

Guzmán B, Vargas P (2005) Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trn*L-*trn*F, and *mat*K sequences. *Molecular Phylogenetics and Evolution* **37**, 644-660.

Hampe A, Arroyo J, Jordano P, Petit RJ (2003) Rangewide phylogeography of a bird-dispersed Eurasian shrub: contrasting Mediterranean and temperate glacial refugia. *Molecular Ecology* **12**, 3415-3426.

Hansen A, Sundig P (1993) Flora of Macaronesia. Checklist of vascular plants. *Sommerfeltia* **17**, 92–105.

Herrera C (1992) Historical effects and sorting processes as explanations for contemporary ecological patterns: character syndromes in Mediterranean woody plants. *The American Naturalist* **140**, 421-446.

Herrera J (1988) Pollination relationships in sourthern Spanish Mediterranean shrublands. *Journal of Ecology* **76**, 274-287.

Hewitt G (2000) The genetic legacy of the Quaternary ice ages. *Nature* **405**, 907-913.

Kelchner SA (2000) The Evolution of non-coding chloroplast DNA and its application in plant systematics. *Annals of the Missouri Botanical Garden* **87**, 482-498.

Krijgsman W, Hilgen FJ, Raffi I, Sierro FJ, Wilson DS (1999) Chronology, causes and progression of the Messinian salinity crisis. *Nature* **400**, 652-655.

Kropf M, Comes HP, Kadereit JW (2006) Long-distance dispersal vs vicariance: the origin and genetic diversity of alpine plants in the Spanish Sierra Nevada. *New Phytologist* **172**, 169-184.

Lavin M, Herendeen PS, Wojciechowski MF (2005) Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the Tertiary. *Systematic Biology* **54**, 575-594.

Linder HP, Hardy CR, Rutschmann F (2005) Taxon sampling effects in molecular clock dating: An example from the African Restionaceae. *Molecular Phylogenetics and Evolution* **35**, 569-582.

Luis-Calabuig E, Tárrega R, Valbuena L (2000) Ten years of recovery of *Cistus ladanifer* after experimental disturbances. *Israel Journal of Plant Sciences* **48**, 271-276.

Lumaret R, Mir C, Michaud H, Raynald V (2002) Phylogeographical variation of chloroplast DNA in holm oak (*Quercus ilex* L.). *Molecular Ecology* **11**, 2327-2336.

Lumaret R, Tryphon-Dionnet M, Michaud H, *et al.* (2005) Phylogeographical variation of chloroplast DNA in Cork Oak (*Quercus suber*). *Annals of Botany* **96**, 853-861.

Magallón SA (2004) Dating lineages: molecular and paleontological approaches to the temporal framework of clades. *International Journal of Plant Sciences* **165**, S7-S21.

Magallón SA, Sanderson MJ (2005) Angiosperm divergence times: The effect of genes, codon positions, and time constraints. *Evolution* **59**, 1653-1670.

Malo JE, Suárez F (1996) *Cistus ladanifer* recruitment - not only fire, but also deer. *Acta Oecologica* **17**, 55-60.

Médail F, Quezel P (1997) Hot-spots analysis for conservation of plant biodiversity in the Mediterranean Basin. *Annals of the Missouri Botanical Garden* **84**, 112-127.

Menke B (1976) Pliozäne und ältestquartäre Sporen- und pollenflora von Schleswig-Holstein. *Geologisches Jahrbuch. Reihe A.* **32**, 3-197.

Mittermeier RA, Robles P, Hoffman M, *et al.* (2004) *Hotspots Revisited* CEMEX, Mexico City.

Montgomery KR, Strid TW (1976) Regeneration of introduced species of *Cistus* after fire in Southern California. *Madroño* **23**, 417-427.

Mort ME, Soltis PS, Soltis DE, Mabry M (2000) Comparison of three methods for estimating internal support on phylogenetic trees. *Systematics Biology* **49**, 160-171.

Naud G, Suc JP (1975) Contribution à l'étude paléofloristique des Coirons (Ardèche). Première analyses polliniques dans les alluvions sous-basaltique et interbasaltiques de Mirabel (Miocène supérieur). *Bulletin de la Société Géologique de France* **17**, 820-827.

Nylander JAA (2002) MrModeltest v1.0b. Department of Systematic Zoology. Uppsala University, Uppsala.

Ortiz MA, Tremetsberger K, Talavera S, Stuessy T, García-Castaño JL (2007) Population structure of *Hypochaeris salzmanniana* DC. (Asteraceae), an endemic species to the Atlantic coast on both sides of the Strait of Gibraltar, in relation to Quaternary sea level changes. *Molecular Ecology* **16**, 541-552.

Ouborg NJ, Piquet Y, van Groenendael JM (1999) Population genetics, molecular markers and the study of dispersal in plants. *Journal of Ecology* **87**, 551-568.

Palibin IV (1909) Cistaceae. In: *Flora Caucasica Critica* (eds. Kuznetsov N, *et al.*), pp. 117-123.

Pérez-García F (1997) Germination of *Cistus ladanifer* seeds in relation to parent material. *Plant Ecology* **133**, 57-62.

Pérez R, Vargas P, Arroyo J (2004) Convergent evolution of flower polymorphism in *Narcissus* (Amaryllidaceae). *New Phytologist* **161**, 235-252.

Petit RJ, Hampe A, Cheddadi R (2005) Climate changes and tree phylogeography in the Mediterranean. *Taxon* **54**, 877-885.

Pons A, Reille M (1988) The Holocene- and upper Pleistocene pollen record from Padul (Granada, Spain): A new study. *Palaeogeography, Palaeoclimatology, Palaeoecology* **66**, 243-263.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Robles C, Bousquet-Mélou A, Garzino S, Bonin G (2003) Comparison of essential oil composition of two varieties of *Cistus ladanifer*. *Biochemical Systematics and Ecology* **31**, 339-343.

Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution* **19**, 101-109.

Simmons MP, Ochoterena H (2000) Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* **49**, 369-381.

Soltis DE, Gitzendanner MA, Strenge DD, Soltis PS (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution* **206**, 353-373.

Suc JP, Bertini A, Combourieu-Nebout N, *et al.* (1995) Structure of West Mediterranean vegetation and climate since 5.3 Ma. *Acta Zoologica Cracoviensia* **38**, 3-16.

Sutherland S, Delph LF (1984) On the importance of male fitness in plants: patterns of fruit-set. *Ecology* **65**, 1093-1104.

Swofford D (2002) *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.* Sinauer, Sunderland, MA.

Talavera S, Gibbs PE, Herrera J (1993) Reproductive biology of *Cistus ladanifer* (Cistaceae). *Plant Systematics and Evolution* **186**, 123-134.

Templeton AR, Crandall KA, Sing CF (1992) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**, 619-633.

Thanos CA, Georghiou K (1988) Ecophysiology of fire-stimulated seed germination in *Cistus incanus* ssp. *creticus* (L.) Heywood and *C. salvifolius* L. *Plant, Cell and Environment* **11**, 841-849.

Thanos CA, Georghiou K, Kadis C, Pantazi C (1992) Cistaceae: A plant family with hard seeds. *Israel Journal of Botany* **41**, 251-263.

Valbuena L, Alonso I, Tárrega R, Luis E (1992) Influencia del calor y del aclarado sobre la germinación de *Cistus laurifolius* y *Cistus ladanifer*. *Pirineos* **140**, 109-118.

Valcárcel V, Fiz O, Vargas P (2003) Chloroplast and nuclear evidence for multiple origins of polyploids and diploids of *Hedera* (Araliaceae) in the Mediterranean basin. *Molecular Phylogenetics and Evolution* **27**, 1-20.

Valdés B (1991) Andalucia and the Rif. Floristic links and a common Flora. *Botanika Chronika* **10**, 117-124.

Van der Pijl L (1979) *Principles of Dispersal in Higher Plants* Springer-Verlag, New York.

Vargas P (2003) Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe. *Taxon* **52**, 463-476.

Vargas P (2007) Are Macaronesian islands refugia of relict plant lineages?: a molecular survey. In: *Phylogeography of Southern European Refugia* (eds. Weiss S, Ferrand N), pp. 297-314. Springer, Dordrecht.

Vargas P, Morton CM, Jury SL (1999) Biogeographic patterns in Mediterranean and Macaronesian species of *Saxifraga* (Saxifragaceae) inferred from phylogenetic analyses of ITS sequences. *American Journal of Botany* **86**, 724-734.

Wang CB, Smith TB (2002) Closing the seed dispersal loop. *Trends in Ecology & Evolution* **17**, 379-385.

Wikström N, Savolainen V, Chase MW (2001) Evolution of the angiosperms: calibrating the family tree. *Proceedings of the Royal Society of London* **268**, 1-10.

Zhang Q, Chiang TY, George M, Liu JQ, Abbott RJ (2005) Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation. *Molecular Ecology* **14**, 3513-3524.

7

Multi-level variation of fruit traits in the multi-valved *Cistus ladanifer* (Cistaceae)

Publicación: Guzmán, B., Narbona, E., Arroyo, J. & Vargas, P. Multi-level variation of fruit traits in the multi-valved *Cistus ladanifer* (Cistaceae) (En preparación)

Abstract

Differences among populations in the expression of characters related to plant fitness could result from natural selection or relaxed selection manifested as phenotypic plasticity in response to local conditions. *Cistus ladanifer* is the unique species in the family (Cistaceae, 180 species) with a variable number of carpels per flower and, thereby valves per fruit. We herein analyse the variation in the number of valves (5-12) and seeds (318-1185) per fruit in 36 populations (607 individuals, 1821 fruits) using a multilevel design: temporal, geographical, ecographical, elevation, taxonomic, and phylogeographical approaches. A marked variation in the number of valves and seeds per fruit among populations, individuals and years was found. Number of ovules and seeds per locule were similar in fruits with different number of fruit valves. Geographical, taxonomic, and phylogeographical variables do not significantly contribute to the variation observed in the number of fruit valves. We have, in contrast, found a negative relationship of number of fruit valves with altitude and positive with precipitation (48% of the total variation). We argue that plasticity, rather than geography, taxonomy and phylogeography, may be involved in multiplication of fruit carpels during the development of the ovary wall, even though the variables studied in the present paper account for a limited proportion.

***Key Words*:** Ecogeographical patterns, elevation, fruit-valve variation, intra-individual variation, inter-individual variation, plasticity, western Mediterranean

1. Introduction

"Isidore Geoffroy Saint-Hilaire insists that, when any part or organ is repeated many times in the same animal, it is particularly liable to vary both in number and structure .... Whenever such parts as the vertebrae or teeth, ..., or petals, stamens, pistils, or seeds, are very numerous, the number is generally variable." —Darwin (1883, chapter XXVI, p. 334).

As flower and fruit characters determine factors involved in fitness (as pollination, dispersal, or predispersal seed predation), they only display slight variation at individual level in response to the different genetic blueprint. On the other hand, vegetative parts tend to be more plastic and variable than reproductive organs due to their different functions in the plant (Stuessy 1990). Floral and fruit features are so conserved that have been used as the essential material for angiosperm classifications (Caesalpinus 1583; Linnaeus 1753). For instances, fruit characteristics are used in delimitation of species of the genus *Valerianella* (Coode 1967) and the number of capsule valves is used in segregating genera in the Caryophyllaceae (*Melandrium, Silene, Cerastium*) (Talavera 1987). Accordingly to this, little variation is expected in flower key characters, which are directly involved in the first stages of the reproductive success.

The Cistaceae comprises eight genera and about 180 species (Arrington & Kubitzki 2003) showing a syncarpous or eusyncarpous 3-carpellated gynoecium with numerous ovules inside. Mature fruits in the Cistaceae have three opened parts (valves), which result from development of three carpels. The only exception for this pattern is *Cistus*, which develops primarily 5-valved fruits. In addition, there exist a single species of *Cistus* (*C. ladanifer)* with a variable number of carpels and then fruit valves (6-12) (Demoly & Montserrat 1993). This variable character is not only unique in the Cistaceae but also rare in the angiosperms, where structural parts of flowers and fruits are profoundly stable within species (Takhtajan 1981; Endress 1994). Nandi (1998a; 1998b) observed numerous flower characters displaying limited variation in the Cistaceae, but ovary divisions variable in *Cistus*. *Cistus ladanifer* constitutes then a remarkable species model to explore multiplication of fruit valves during the development of the ovary wall.

A historical reconstruction of the evolution of the number of fruit valves within the Cistaceae clearly showed a transition from fruits divided in three to five or more valves (Guzmán & Vargas 2005). The gum rock-rose (*Cistus ladanifer*) produces a high number of flowers and fruits at the apex of each branch of the last year depending on particular conditions (Talavera *et al.* 1993), but causes behind variation in number of valves remain elusive. Ecogeography accounts for numerous patterns of plant differentiation at the species level, particularly when populations cope with complex geography and ecology (Endler 1986; Cadaval 1999; Silva-Montellano & Eguiarte 2003; Petru *et al.* 2006). The gum rock-rose is in fact a woody perennial shrub occurring in a wide range of sites on European and African sides of the western Mediterranean. The above mentioned characteristics make *C. ladanifer* especially suitable to investigate trends in fruit variation across the species distribution. A multi-scaled approach may allow us distinguishing between the contribution of genetic/developmental constraints and spatial conditions.

Despite variation in multiple valves is a rare character within a single species, little attention has been paid to explore the causes behind variation in *C. ladanifer*. The aim of the present work is to assess the variability in the number of valves and seeds per fruit in *Cistus ladanifer* and investigate the role of ecogeographical factors. Specifically, we address the following main questions: (1) is the number of valves variable within individuals?; (2) are there trade-offs between the production of ovules and seeds per valve and the number of valves per fruit?; (3) is there significant variation in the number of valves and seeds per fruit between and within populations?; (4) is this variation constant through time?; (5) are geographical and ecographical conditions involved?; (6) at what extent fruit variation is correlated with taxonomy and phylogeography?

2. Material and Methods

2.1. Study species

Cistus ladanifer L. is a western Mediterranean shrubby species occurring in a wide range of latitude (33° to 43°), altitude (0 to 1500 m), climate (dry to humid) and have a geographic distribution in southern France, Portugal, Spain, northern Morocco and northern Algeria (Demoly & Montserrat 1993) (Fig. 1A). This entomophilous species colonizes degraded areas and its abundance has increased in the last decades due to human disturbance (Trabaud 1995; Luis-Calabuig *et al.* 1996).

Morphological variation in vegetative characters of *Cistus ladanifer* is recognized into three subspecies: *ladanifer*, distributed in Spain, Portugal, France and northern Morocco; *africanus*, distributed in southern Spain (Cádiz and Málaga), northern Morocco and northern Algeria; and *sulcatus*, endemic to the southwest Portugal (Algarve region) (Demoly & Montserrat 1993).

The fruits of *C. ladanifer* are globular lignified capsules with 6-12 valves (Fig. 2), which produce a large number of seeds that remain inside the capsules until summer, when fruit valves dehiscence and seeds fall near the mother plant (Bastida & Talavera 2002) (Fig. 2). The gum rock-rose is an obligatory-seeder plant so, after an intense disturbance such fire, the population is regenerated through the soil seed bank (Montgomery & Strid 1976; Arianoutsou & Margaris 1981; Valbuena *et al.* 1992).

The study was carried out during 2003-2006 in 36 populations, mostly covering the species distribution area (Appendix 1, Fig. 1B, 1C, 1D). All the study locations were natural patches where *C. ladanifer* was the dominant species.

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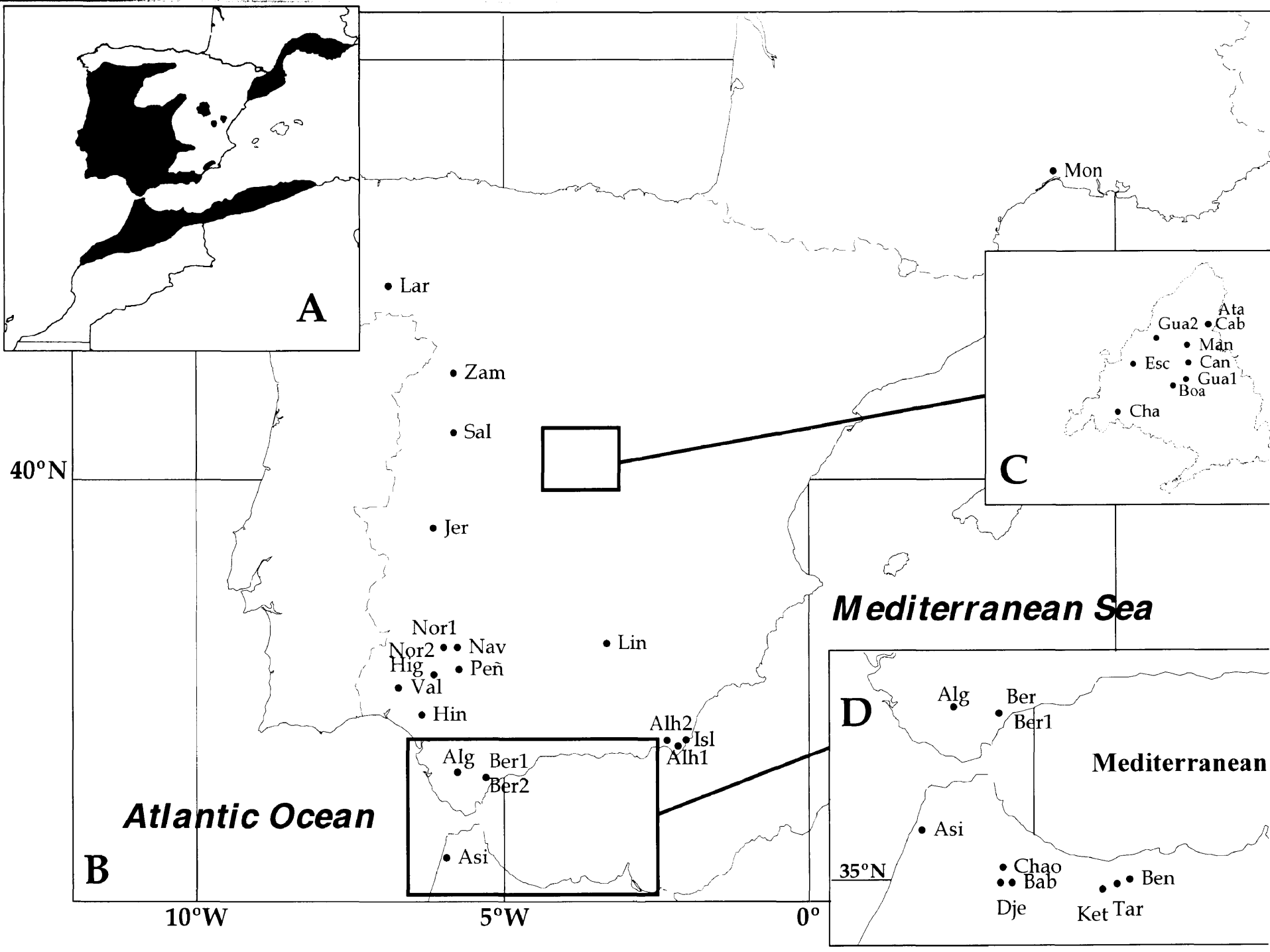
Fig. 1. Distribution area (A) and geographical location of the 36 studied populations of *C. ladanifer* from Portugal, Spain, France and northern Morocco (B). Detail of Madrid province limits (C), and the Strait of Gibraltar region (D). Population abbreviations as in Appendix 1.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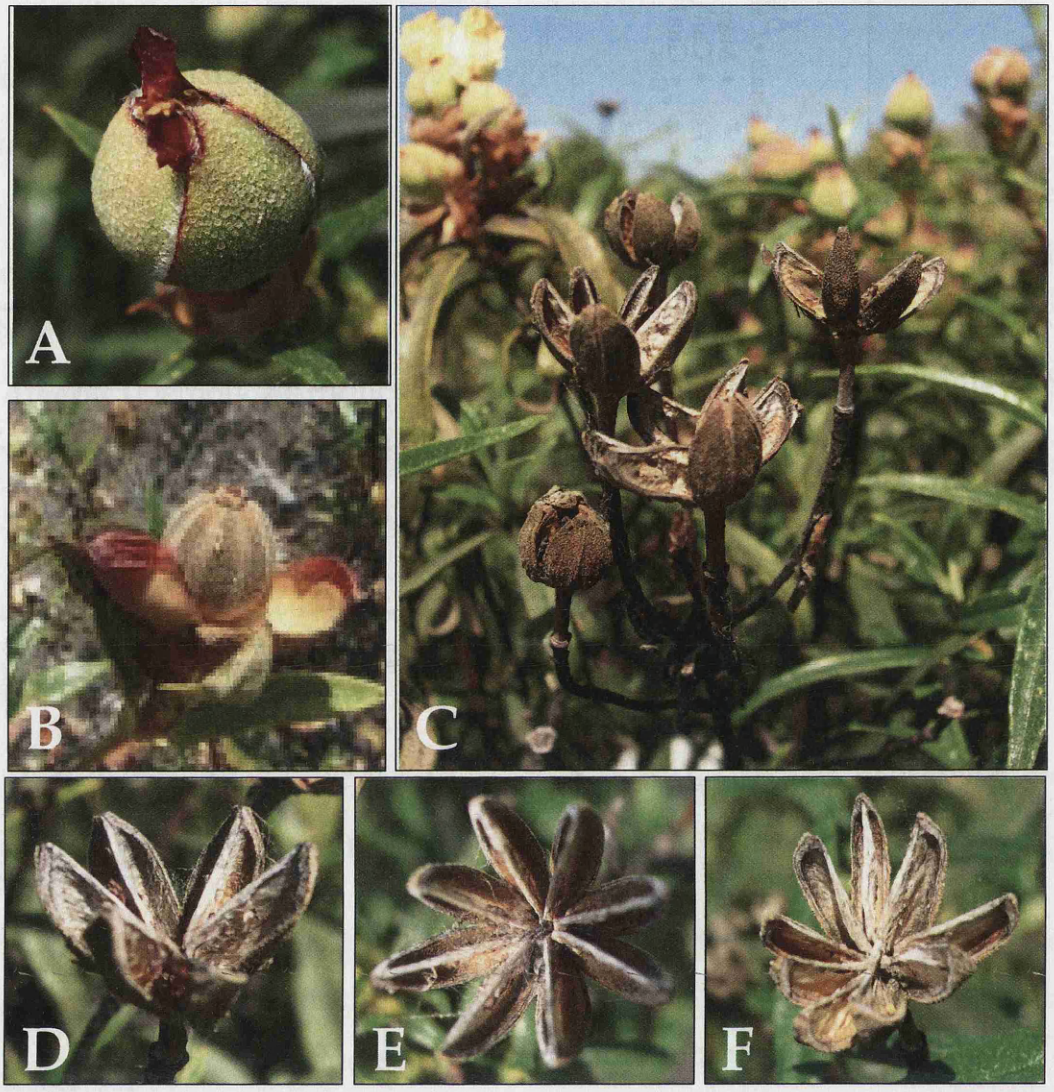

Fig. 2. *Cistus ladanifer* fruits. (A) Immature capsule covered by the sepals. (B) Mature capsule with falling sepals. (C) Infrutescence of mature and opened capsules at the end of woody pedicels. Opened capsules with six (D), eight (E), and nine valves (F). All photographs by Beatriz Guzmán.

2.2. Sampling

Geographical level. To explore the existence of a geographical pattern in the variation of the number of fruit valves, 34 populations were analysed (Table 1). In each population two-four fruits between six and 21 individuals each were randomly recollected to count the number of valves. A subset of 19 populations was chosen to analyse the variability in the number of seeds per fruit (Table 1). We estimated the number of seeds per fruit

Table 1. Population number, population identity and number of individuals studied of *C. ladanifer*. Populations codes as in Appendix 1

STUDY LEVEL	NUMBER OF POPULATIONS	NUMBER OF INDIVIDUALS PER POPULATION	POPULATIONS CODE
VALVE-NUMBER			
Geographical	34	6-21	All populations except Ber2, Nor2
Ecogeographical	34	6-21	All populations except Ber2, Nor2
Elevation	8	40	Gua1, Gua2, Alh1, Alh2, Ber1, Ber2, Nor1, Nor2
Temporal and Intra- interpopulation (2 years)	19	6-20	Lar, Sal, Ata, Cab, Man, Esc, Can, Gua1, Gua2, Boa, Cha, Jer, Nor1, Hig, Val, Alh2, Isl, Alh1, Ber
Temporal and Intra- interpopulation (3 years)	6	20-40	Can, Gua, Boa, Cha, Alh1, Alh2
Taxonomic	34	6-21	All populations except Ber2, Nor2
Phylogeographical	24	7-20	Mon, Lar, Zam, Sal, Ata, Cab, Man, Esc, Gua1, Gua2, Boa, Cha, Jer, Nor1, Hin, Alh2, Isl, Ber, Asi, Bab, Dje, Ben, Tar, Ket
SEEDS-NUMBER			
Geographical	19	4-16	Lar, Zam, Ata, Cab, Man, Esc, Boa, Cha, Lin, Nav, Nor1, Peñ, Val, Hin, Alh2, Ber, Chao, Bab, Ket
Ecogeographical	19	4-16	Lar, Zam, Ata, Cab, Man, Esc, Boa, Cha, Lin, Nav, Nor1, Peñ, Val, Hin, Alh2, Ber1, Chao, Bab, Ket
Taxonomic	19	4-16	Lar, Zam, Ata, Cab, Man, Esc, Boa, Cha, Lin, Nav, Nor1, Peñ, Val, Hin, Alh2, Ber1, Chao, Bab, Ket
TRADE OFF			
Ovules/valve	4	20	Cab, Esc, Nor1, Ber1
Seeds/valve	4	20	Cab, Esc, Nor1, Ber1

by counting two opposite valves, and multiplying the mean number by the number of valves of one-three fruits in four-sixteen individuals (Table 1).

Ecographical level. We analyse any relationship between environmental factors (altitude, mean annual temperature, mean maximum temperature of the hottest month, mean annual precipitation) (Appendix 1) and the number of valves and seeds per fruit counted on the same populations as in the geographical level (Table 1).

Elevation level. We explore if a pattern related with population altitude in the number of valves and seeds per fruit existed. To avoid a possible geographical effect we selected four Iberian mountains (Guadarrama, Alhamilla, Bermeja, Norte of Seville). In each of them, we chose two populations covering the maximum range of altitude (240-1400 m) of the species distribution in the four mountain ranges: Guadarrama (Gua1, Gua2); Alhamilla (Alh,1, Alh2); Bermeja (Ber1, Ber2); Norte of Seville (Nor1, Nor2) (Appendix 1, Table 1). We randomly selected 40 individuals and four fruits per individual from each population.

Temporal level. To investigate whether the number of fruit valves was constant across years, 26 individuals from 19 Iberian populations (Table 1) were randomly selected in 2004 and 2005. In each individual, four fruits on branches facing the four geographic points were randomly recollected to count the number of valves. A subset of six widely distributed populations was selected to perform the same study on the same individuals in 2006 (Table 1).

Taxonomic level. We explore whether the number of valves and seeds vary between *C. ladanifer* subspecies. We employed the data of 27 populations of the subsp. *ladanifer* and seven of subsp. *africanus* to analyse variability in number of valves, and a subset of 16 populations of subsp. *ladanifer* and three of subsp. *africanus* to study the variability in the number of seeds per fruit (Appendix 1, Table 1).

Phylogeographical level. We analyse any relationship between variation in the number of fruit valves and the haplotypes found in *C. ladanifer* (Guzmán & Vargas

unpublished). The sequencing of the plastid *trnS-G* and *trnK-matK* spacers revealed eight haplotypes in 24 populations (Appendix 1, Table 1). "Taf" population was not included in the analysis because is the only population with haplotype 7. Similarly, we did not analyse the relation between haplotypes and the number of seeds per fruit because all the populations showed the same haplotype (number one) except for "Ber" with haplotype 6 and "Ket" with haplotype 4 because violating replicate requirements.

Trade-off. To investigate if there are compensatory effects (trade-offs) between the production of ovules and seeds per valve and the number of valves in each fruit, we counted the number of seeds and aborted ovules in two opposite valves of three-five fruits in 20 individuals of four populations (Table 1).

2.3. *Statistical analyses*

Prior to analysing the variability at different levels, we tested whether the number of valves and seeds per fruit varied between populations using one-way ANOVA considering "population" as a random factor. Differences in the number of fruit valves among populations, individuals and years were tested using an ANOVA for repeated measures, in which the factor "population" was considered random and "individual", considered also random, was nested within population. Spatial statistics were used to explore if variation between populations in number of valves and seeds per fruit could be explained through a geographical pattern. The spatial autocorrelation in the two variables was explored by means of semivariograms using the software GS+ 5.0 (Gamma Design Software, Plainville, Michigan, USA). In the semivariogram, the proportion of total variance explained by spatial dependence is expressed by the structural fraction and the fit of the model to the semivariance analysis results is assessed in terms of the model r^2. To test the relationship between the number of valves and seeds per fruit and the four ecogeographical variables (ALTITUDE, T, T_h, PREC; Appendix 1) a multiple regression analysis was carried out. The number of fruit valves of the low and high populations from four mountains was compared using one-way ANOVAs in which the factor "altitude" was considered fixed. Variation of number of

valves and seeds per fruit between subspecies were analysed using mixed-model ANOVAs, in which the factor "subspecies" was fixed and the factor "population" was random and nested within subspecies. The same analysis was performed to determine differences in number of valves and seeds per fruit between haplotypes. One-way ANOVAs were also performed to explore the existence of a compensatory effect.

When the ANOVA showed significant differences, the mean of groups were compared using the post-hoc Tukey HSD tests since the variance between groups was homogenous and unplanned comparisons were to be made (Day & Quinn 1989). Prior to any analysis, normality of variables was checked with Kolmogorov-Smirnov test and homocedasticity with Levene's test (Day & Quinn 1989). The number of seeds per fruit was logarithm (base 10) transformed. The number of valves per fruit could not be transformed to a normal distribution, but F-test is robust to deviations from normality when N is sufficiently large (Lindman 1974; Zar 1999). In the graphical representations we employed a bootstrap method to calculate confidence intervals of variables with heterogeneous variances. We employed 100,000 measurements and a confidence probability of 0.95 to obtain the intervals. To control for the experiment wise type I error produced by multiple comparisons, we applied the sequential Bonferroni test for fitting the significance level (Rice 1989). All the statistical analyses were performed using the computer program STATISTICA 6.0 (Statsoft Inc., Tulsa, USA), except for the bootstrapping estimation which was performed with DataPilot 1.03, a macro for Excel (TwoPilot Inc.).

3. Results

The number of valves per fruit was highly variable in the 36 studied populations, ranged from 5 (populations Alh1, Alh2, Gua1, Gua2) to 12 (Bab1). The mean population number of fruit valves ranged from 7.2 ± 0.04 (Alh1) to 10.0 ± 0.00 (Chao, Peñ) (Fig. 3A). The mean population number of seeds per fruit ranged from 318.7 ± 55.83 (Alh2) to 1364.3 ± 63.14 (Nor1) (Fig. 3B). Both mean numbers of fruit valves and seeds per fruit

differed significantly among populations ($F_{35, 571}$ = 19.30, P < 0.0001 and $F_{20, 223}$ = 20.2, P < 0.0001; respectively).

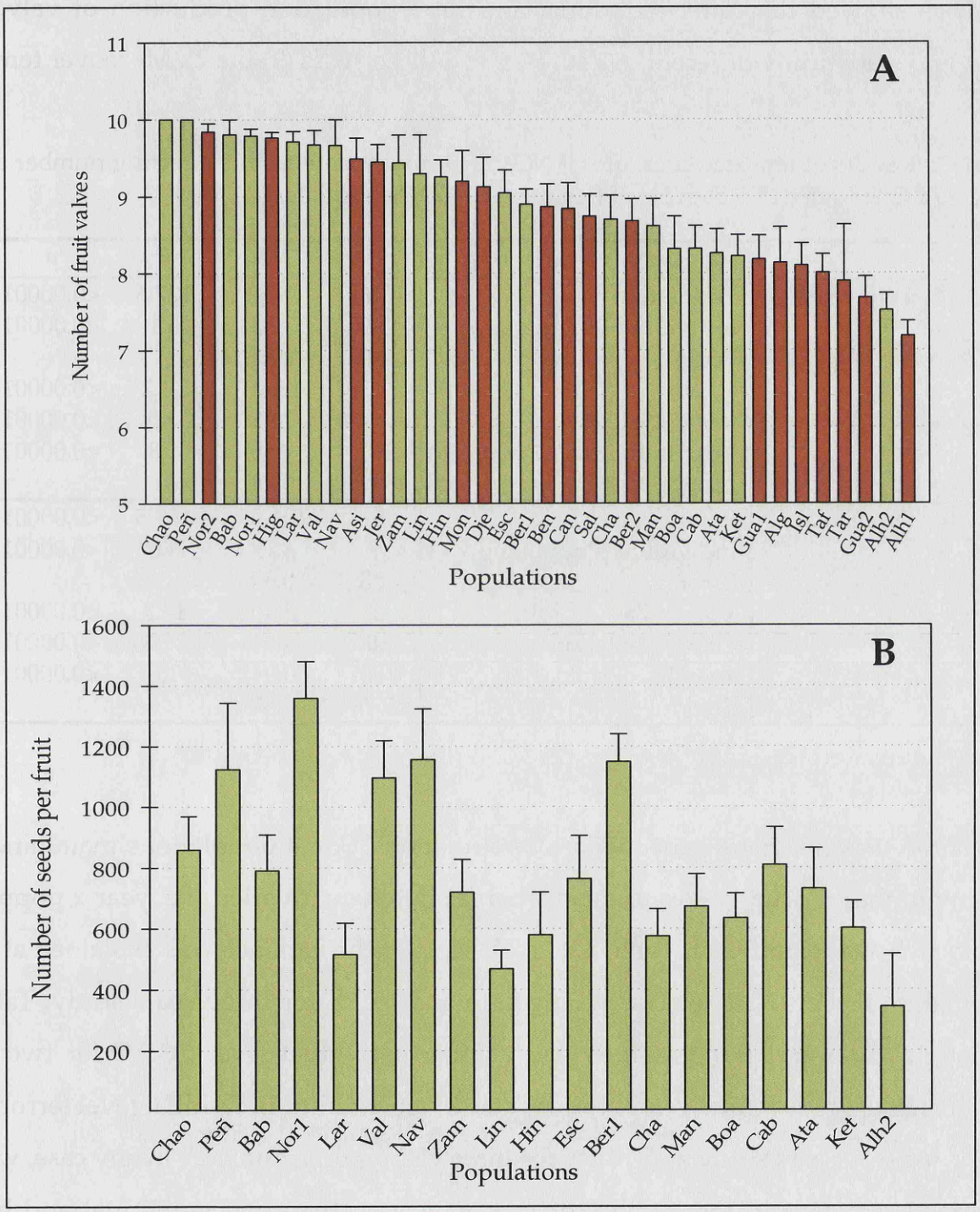

Fig. 3. Mean values (± confidence interval) of the number of fruit valves (A) and seeds (B) per fruit in populations of *C. ladanifer* distributed in south-eastern Europe and northern Africa (P < 0.05, estimated by means of bootstrapping, 100,000 runs). See Appendix 1 for population codes.

The number of fruit valves differed significantly not only between individuals and populations but also within the same individual (Table 2). The repeated measures analysis showed that inter-individual and inter-population production of valves per fruit was statistically different in both two and three years (Table 2). Moreover temporal

Table 2. Results of repeated-measures ANOVA for investigate factors affecting number of fruit valves of *C. ladanifer*. *df* = degrees of freedom; *MS* = Mean squares

	Source of variation	*df*	*MS*	*F*	*p*
A. Two years	Population	18	69.31	127.6	<0.00001
	Individual (Population)	299	3.33	6.1	<0.00001
	Error	889	0.54		
	Year	1	16.10	33.2	<0.00001
	Year*Population	18	2.91	6.0	<0.00001
	Year*Individual (Pop.)	299	0.88	1.8	<0.00001
	Error	889	0.48		
B. Three years	Population	5	101.96	169.5	<0.00001
	Individual (Population)	93	5.19	8.6	<0.00001
	Error	288	0.60		
	Year	2	8.40	14.8	<0.00001
	Year*Population	10	4.51	7.9	<0.00001
	Year*Individual (Pop.)	186	0.94	1.6	<0.00001
	Error	576	0.57		

variation was not homogeneous in all populations. Some populations maintained the mean number of fruit valves in time, whereas in others it varied (i.e. year x population interaction was significant, Table 2, Fig. 4). Most of the variance was explained at inter-population level (41.2% for two years study and 69.7% for three years study; Table 2), although some variation was detected at inter-individual level (33.2% for two years study and 19.9% for three years study; Table 2) and at intra-individual level (error term; 25.6% for two years study and 10.4% for three years study; Table 2). In any case, we also observed variation in the number of fruit valves on the same individual and branch across years (results not shown).

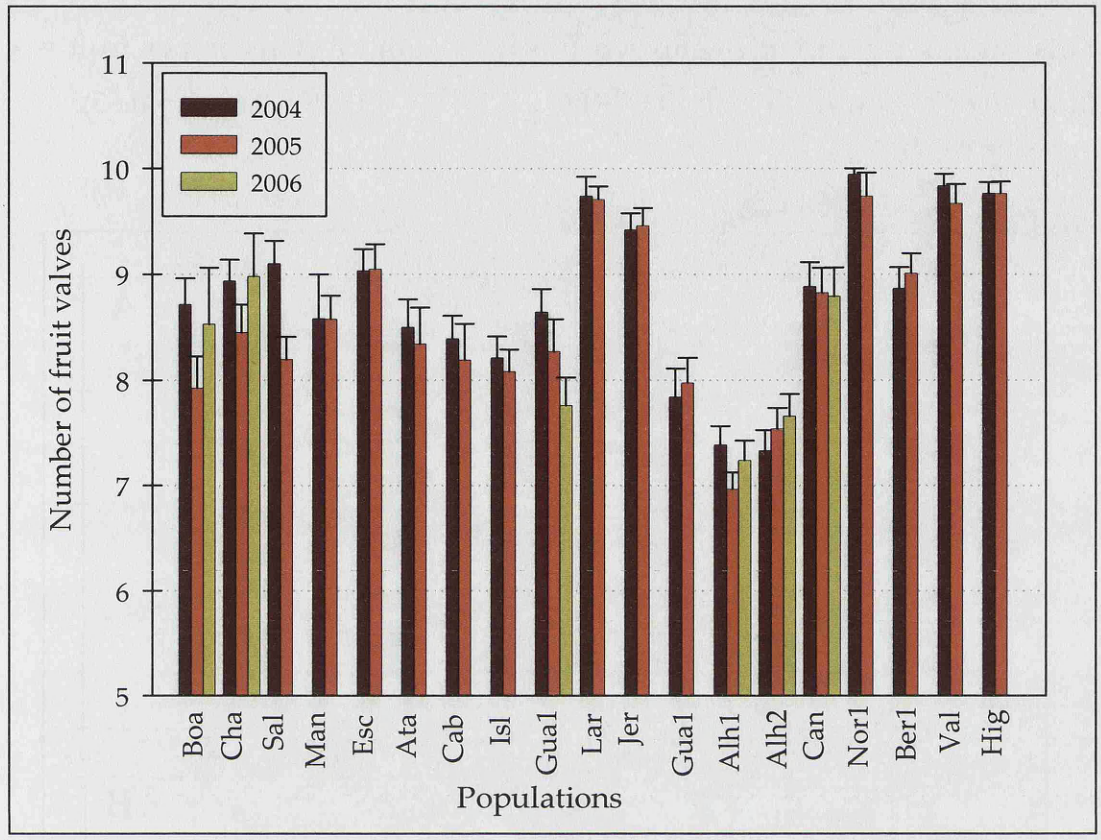

Fig. 4. Variability in mean number of fruit valves (± confidence interval) through time ($P < 0.05$, estimated by means of bootstrapping, 100,000 runs). See Appendix 1 for population codes.

The semivariance analyses showed that variables did not fit accurately to the theoretical semivariogram models. The semivariogram data for the log number of seeds per fruit were: model = Gaussian; spatial structure = 0.86; $r^2 = 0.17$. In the case of the number of fruit valves the fit to the theoretical model was better (model: Spherical; spatial structure = 0.72; $r^2 = 0.40$) but not enough to ensure a significant spatial structure.

The multiple regression analysis carried out with the number of fruit valves and the ecographical variables was statistically significant and showed a moderate predictive power (adjusted $r^2 = 0.45$). The analysis showed that altitude and mean annual precipitation explained the 48% of the total variance (regression function: number of fruit valves = - 0.51 * altitude + 0.52 * PREC + 8.78; $r^2 = 0.48$; $F = 13.52$; $P = 0.00007$) (Fig.

5). However, the multiple regression analysis carried out with the seeds per fruit was not statistically significant (regression function: number of seeds per fruit = - 0.39 * altitude - 0.63 * T + 0.42 * T_h - 0.29 * PREC + 4.39; $r^2 = 0.15$; F = 0.62; $P = 0.65$).

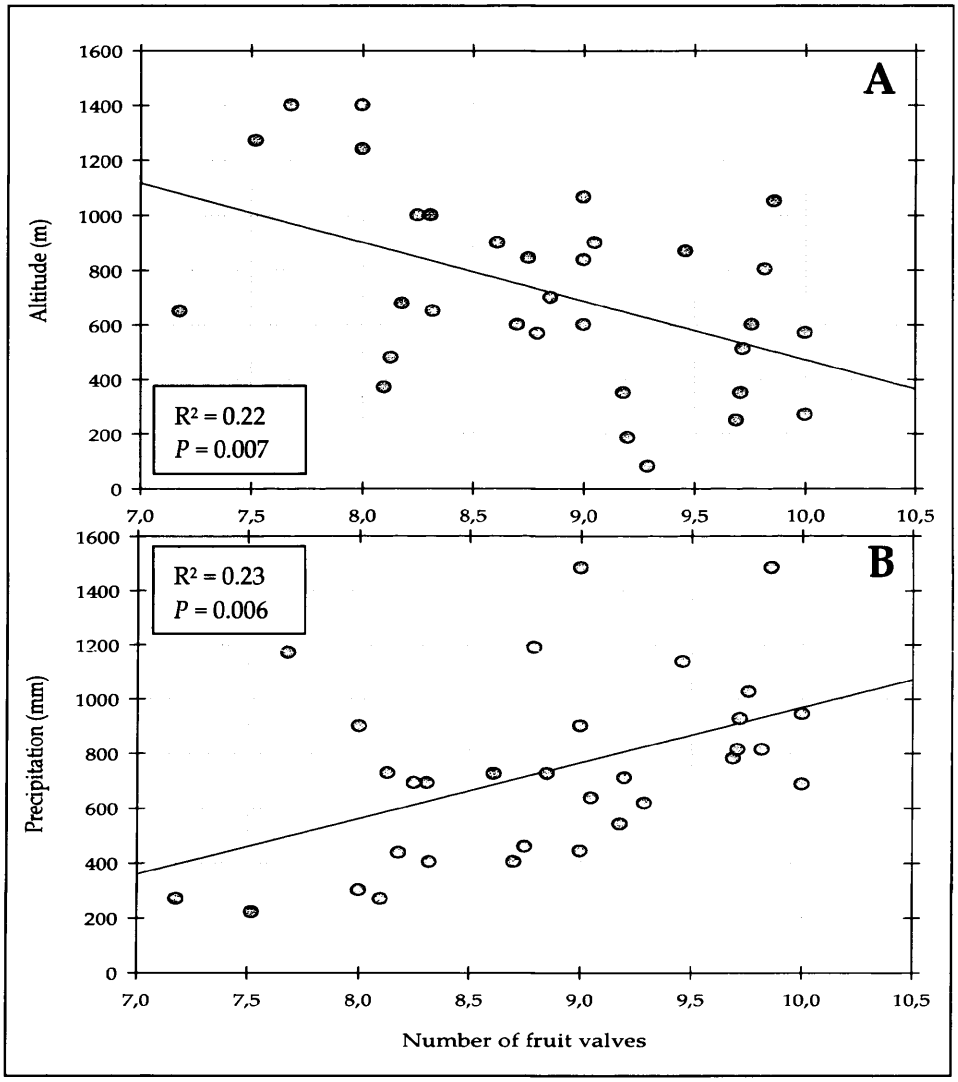

Fig. 5. Relationship between number of fruit valves and two ecogeographical variables: altitude (A) and mean annual precipitation (B).

In two of the four studied Iberian mountains (Bermeja, Guadarrama), the number of fruit valves statistically decreased in the high populations (Fig. 6, Table 3). In Sierra Norte of Seville, there were no significant differences between the low/high altitude populations, and in Alhamilla Mountain the high population produced a higher number of fruit valves although differences were marginally significant (Table 3, Fig. 6).

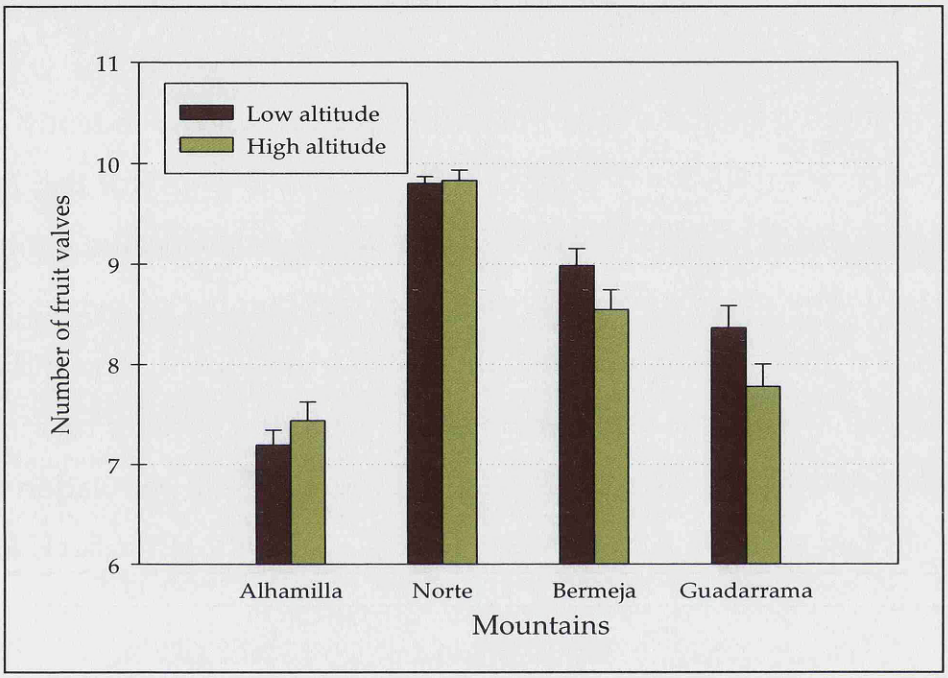

Fig. 6. Population mean and confidence interval of the number of fruit valves studied in the altitude study ($P < 0.05$, estimated by means of bootstrapping, 100,000 runs).

Table 3. Results of one-way ANOVA comparing the number of fruit valves from two populations covering the range of distribution of *C. ladanifer* in four Iberian mountains. *df* = degrees of freedom; *MS* = Mean squares

		df	*MS*	*F*	*p*
A. Alhamilla	Elevation	1	1.17	3.76	0.06
	Error	78	0.31		
B. Bermeja	Elevation	1	3.78	10.44	0.002
	Error	78	0.36		
C. Guadarrama	Elevation	1	7.02	13.05	0.0005
	Error	78	0.54		
D. North of Seville	Elevation	1	0.02	0.22	0.64
	Error	77	0.08		

The two subspecies of *C. ladanifer* produced fruits with a similar number of valves (8.8 ± 0.05 for subsp. *ladanifer*, 8.8 ± 0.08 for subsp. *africanus*) and seeds per fruit (833.12 ± 29.27 for subsp. *ladanifer*, 1018.43 ± 44.72 for subsp. *africanus*), but again differences between populations were statistically significant (Table 4). Similarly, the number of fruit valves of different haplotypes of *C. ladanifer* was statistically similar (8.8 ± 0.05 for haplotype 1, 8.7 ± 0.16 for haplotype 4, 8.2 ± 0.1 for haplotype 6) (Table 4).

Fruits produced a similar number of ovules per valve independently of the number of fruit valves ($F_{5,90} = 0.20$, $P = 0.96$ for Cab; $F_{4,94} = 0.48$, $P = 0.75$ for Esc; $F_{3,90} = 1.38$, $P = 0.25$ for Nor1), except in Ber1 where differences were statistically significant ($F_{3,106} = 6.06$, $P = 0.007$). The post hoc test revealed that the number of ovules per valve was higher in fruits with eight valves than in the rest of fruits (seven, nine and ten valves). A compensatory effect between the seeds per valve and the number of fruit valves does not exist, since statistically differences were not found in any population ($F_{5,90} = 0.38$, $P = 0.85$ for Cab; $F_{4,94} = 0.85$, $P = 0.94$ for Esc; $F_{3,106} = 2.02$, $P = 0.11$ for Ber1; $F_{3,90} = 0.28$, $P = 0.83$ for Nor1).

4. Discussion

The gum rock-rose (*Cistus ladanifer*) presented great variation in fruit characters. The number of valves per fruit (5-12) was variable at the inter-population, inter-individual and intra-individual levels. Although different sources of variation are involved, most of the heterogeneity was detected among populations (41.2% in the two years study; 69.7% in the three years study). The same is true for an inconsistent level of fruit-valve variation in time. Some individuals produced the same number of fruit valves in successive years, whereas others produced a different number. The same inconsistency is applicable at the population level (Table 2). To our knowledge, this is one of the first studies reporting an extraordinary variation in valves per fruit within a single species and over time. Acosta *et al.* (1993) found similar levels of variation in *C. ladanifer* at the inter- and intra-individual levels, but their study was carry out in only one population. In *Helleborus foetidus* (Ranunculaceae), a high variation in the number of carpels was

Table 4. Results of nested ANOVA to investigate the effects of subspecies and haplotypes on number of fruit valves and number of seeds per fruit in populations of *C. ladanifer* sampled in Morocco, Portugal, Spain and France. *df* = degrees of freedom; *MS* = Mean squares.

	Source of variation	NUMBER OF VALVES				LOG NUMBER OF SEEDS			
		df	*MS*	*F*	*p*	*df*	*MS*	*F*	*p*
A. Taxonomy	Subspecies	1	5.79	0.25	0.62	1	0.12	0.43	0.52
	Population (Subspecies)	32	25.19	1.62	0.02	17	4.47	19.73	<
	Error	533	15.50			185	2.46		0.0001
B. Phylogeography	Haplotype	2	10.79	1.50	0.25				
	Population (Haplotype)	20	7.64	12.96	< 0.0001				
	Error	378	0.60						

found at the intra- and inter-population levels, but the magnitude of the variation (2-4 carpels) was lower (Guitián *et al.* 2003). Though difficult to interpret, the remarkable variation in fruit features of *C. ladanifer* may have important consequences in the maternal fitness as discussed in *Helleborus foetidus* (Alcántara *et al.* 2007).

At a geographic scale, the number of valves per fruit does not show a pattern of spatial variation (spatial structure = 0.72, r^2 = 0.40). The most important geographical feature in the distribution area of the gum rock-rose (the Strait of Gibraltar) appears not to originate differentiation processes related to the variation in numbers of valves and seeds. This result is, in part, congruent with the at least two migrations of *C. ladanifer* from Africa to Europe (Guzmán & Vargas unpublished). We have found that the number of fruit valves was similar between haplotypes (8.8 ± 0.05 for haplotype 1, 8.7 ± 0.16 for haplotype 4, 8.2 ± 0.1 for haplotype 6), indicating that differences among populations are not due to coancestry patterns or genetic relationships among populations. For instance, the taxonomic delimitation of *C. ladanifer* in subsp. *africanus* is mostly congruent with haplotype distribution (but not with variation in fruit valves; 8.8 ± 0.05 for subsp. *ladanifer*, 8.8 ± 0.08 for subsp. *africanus*), as the four haplotypes of this subspecies form a well-supported clade (Guzmán & Vargas unpublished). Further molecular studies using more variable molecular markers (such as AFLPs) may help elucidate fine scale genetic diversity (but see below for *C. albidus*).

The correlation study between the number of fruit valves and four environmental factors showed a subtle, but statistically significant correlation. High altitude and low precipitation are correlated with a considerable lower number of fruit valves (Fig. 5). The wide distribution area of *C. ladanifer* (Fig. 1A) offers exposures to a broad range of environmental conditions (Joshi *et al.* 2001). In order to persist in different environments, the gum rock-rose may have cope with particular abilities to vary the expression of some morphological traits. In fact, Nuñez-Olivera *et al.* (1996) found that *C. ladanifer* leaves were very plastic on relation with precipitation. In the closely-related *C. salviifolius* (Guzmán & Vargas 2005), leaf length has been correlated with precipitation while geographic longitude explains leaf width and internode length

variation (Farley & McNeilly 2000). In particular, populations of *C. salviifolius* from highly different habitats showed a similar genetic structure. In *C. albidus*, there is no particular genotype associated to different environmental conditions (high temperature or low rainfall), suggesting that phenotypic plasticity, rather than natural selection on genotypes, is responsible for *C. albidus* phenotypes occurring in different environmental conditions (Grant *et al.* 2006). Similarly, high multiplication of valves in *C. ladanifer* in relation to high altitude and low precipitation conditions could reflect the high degree of phenotypic plasticity of this reproductive feature. The fact that the number of fruit valves of individuals and populations was highly variable across years and not linked to plastid haplotype lineages supports this hypothesis.

Climate determines the geographical range of many species (Woodward & Williams 1987; Pigott 1992). As environmental factors often change in a clinal manner (Endler 1977), the favourability of a species environment decreased from the core to the periphery (Brown 1984; Jump & Woodward 2003). Both, plant growth and reproduction are expected to decrease when environmental favourability declines (Hengeveld & Haeck 1982; Parsons 1991), consequently many species exhibit a lower seed production toward their range boundary (Pigott & Huntley 1981; García *et al.* 2000; Dorken & Eckert 2001). In *C. ladanifer*, we have found a tendency to decrease the number of fruit valves in peripheral populations upon the harsh habitats found at high altitudes and dry soils (Fig. 5). The reduction in the reproductive potential at the population level due to the variation in fruit structure (less number of fruit valves) could be the sign of the environmental factors determined by altitudinal limits in the distribution area of the gum rock-rose. On the other hand, and similarly to that found in *Helleborus foetidus* (Guitián et al. 2003), we did not find a trade-off between number of ovules and seeds per valve with different numbers of valves per fruit ($F_{5,90} = 0.38$, $P = 0.85$ for Cab; $F_{4,94} = 0.85$, $P = 0.94$ for Esc; $F_{3,106} = 2.02$, $P = 0.11$ for Ber1; $F_{3,90} = 0.28$, $P = 0.83$ for Nor1). Fruits with more valves accordingly produce a higher number of seeds. However, we did not find a relationship between production of seeds and any of the environmental variables. We argue that plasticity, rather than geography, phylogeography or taxonomy, may be

involved in multiplication of fruit carpels during the development of the ovary wall, even though the variables studied in the present paper account for a limited proportion.

Our study supports early predictions by Saint-Hilaire and Darwin as multiplication of flower parts being "...very numerous, the number is generally variable" (Darwin 1859). Further developmental, genetic and ecological studies are needed to elucidate the causes of the extraordinary variability found in the number of fruit valves in the gum rock-rose. *Ex situ* studies under controlled (common garden) conditions, coupled with analysis of fruit gene expression, are necessary to determine differential selection between populations given that they occur in highly heterogeneous environments.

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References

Acosta FJ, Serrano JM, Pastor C, López F (1993) Significant potential levels of hierarchical phenotypic selection in a woody perennial plant, *Cistus ladanifer*. *Oikos* **68**, 267-272.

Alcántara JM, Rey PJ, Manzaneda JA, *et al.* (2007) Geographic variation in the adaptive landscape for seed size at dispersal in the myrmecochorous *Helleborus foetidus*. *Evolutionary Ecology* **21**, 411-430.

Arianoutsou M, Margaris NS (1981) Early stages of regeneration after fire in a phryganic ecosystem (east Mediterranean). I. Regeneration by seed germination. *Biologie et écologie méditerranéenne* **8**, 119-128.

Arrington JM, Kubitzki K (2003) Cistaceae. In: *The Families and Genera of Vascular Plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales.* (ed. Kubitzki K), pp. 62-70. Springer, Berlin.

Bastida F, Talavera S (2002) Temporal and spatial patterns of seed dispersal in two *Cistus* species (Cistaceae). *Annals of Botany* **89**, 427-434.

Brown JH (1984) On the relationship between abundance and distribution of species. *American Naturalist* **124**, 255-279.

Cadaval A (1999) *Estudio evolutivo de los azúcares del néctar de Agave lechuguilla en el desierto de Chihuahua*, Universidad Nacional Autónoma de México.

Caesalpinus A (1583) *De plantis libri XVI*, Florence.

Coode MJE (1967) Materials towards a Flora of Turkey XV. *Notes of the Royal Botanical Garden of Edinburgh* **27**, 219–256.

Darwin C (1859) *The Origin of Species by Means of Natural Selection*, London.

Darwin C (1883) *The variation of animals and plants under domestication* D. Appleton & Co. , New York, USA.

Day RW, Quinn GP (1989) Comparisons of treatments after an analysis of variance in ecology. *Ecological Monographs* **59**, 433-463.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Dorken ME, Eckert CG (2001) Severely Reduced Sexual Reproduction in Northern Populations of a Clonal Plant, *Decodon verticillatus* (Lythraceae). *The Journal of Ecology* **89**, 339-350.

Endler JA (1977) *Geographic variation, speciation, and clines* Princeton University Press, Princeton, New Jersey.

Endler JA (1986) *Natural selection in the wild* Princeton University Press, Princeton, New Jersey.

Endress PK (1994) *Diversity and evolutionary biology of tropical flowers* Cambridge University Press, Cambridge.

Farley RA, McNeilly T (2000) Diversity and divergence in *Cistus salviifolius* (L.) populations from contrasting habitats. *Hereditas* **132**, 183-192.

García D, Zamora R, Gomez JM, Jordano P, Hodar JA (2000) Geographical variation in seed production, predation and abortion in *Juniperus communis* throughout its range in Europe. *Journal of Ecology* **88**, 436-446.

Grant OM, McNeilly T, Incoll LD (2006) Genetic diversity of *Cistus albidus* in south-east Spain does not relate to mesoclimate. *Functional Plant Biology* **33**, 247-255.

Guitián J, Medrano JM, Herrera CM, Sánchez-Lafuente AM (2003) Variation in structural gender in the hermaphrodite *Helleborus foetidus* (Ranunculaceae): within- and among-population patterns. *Plant Systematics and Evolution* **241**, 139-151.

Guzmán B, Vargas P (2005) Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trn*L-*trn*F, and *mat*K sequences. *Molecular Phylogenetics and Evolution* **37**, 644-660.

Hengeveld R, Haeck J (1982) The distribution of abundance. 1. Measurements. *Journal of Biogeography* **9**, 303-316.

Joshi J, Schmid B, Caldeira MC, *et al.* (2001) Local adaptation enhances performance of common plant species. *Ecology Letters* **4**, 536-544.

Jump AS, Woodward FI (2003) Seed production and population density decline approaching the range-edge of *Cirsium* species. *New Phytologist* **160**, 349-358.

Lindman HR (1974) *Analysis of variance in complex experimental designs* W.H. Freeman & Co, San Francisco.

Linnaeus C (1753) *Species Plantarum*, Estocolmo.

Luis-Calabuig E, Tárrega R, Alonso I (1996) Seedling regeneration of 2 *Cistus* species after experimental disturbances. *International Journal of Wildland Fire* **6**, 13-19.

Montgomery KR, Strid TW (1976) Regeneration of introduced species of *Cistus* after fire in Southern California. *Madroño* **23**, 417-427.

Nandi OI (1998a) Floral development and systematics of Cistaceae. *Plant Systematics and Evolution* **212**, 107-134.

Nandi OI (1998b) Ovule and seed anatomy of Cistaceae and related Malvanae. *Plant Systematics and Evolution* **209**, 239-264.

Nuñez-Olivera E, Martinez-Abaigar J, Escudero JC (1996) Adaptability of leaves of *Cistus ladanifer* to widely varying environmental conditions. *Functional Ecology* **10**, 636-646.

Parsons P (1991) Evolutionary rates: stress and species boundaries. *Annual Review of Ecology & Systematics* **22**, 1-18.

Petru M, Tielbörger K, Belkin R, Sternberg M, Jeltsch F (2006) Life history variation in an annual plant under two opposing environmental constraints along an aridity gradient. *Ecography* **29**, 66-74.

Pigott CD (1992) Are the distributions of species determined by failure to set seed? In: *Fruit and seed production: aspects of development, environmental physiology and ecology* (eds. Marshall C, Grace J), pp. 203-216. Cambridge University Press, Cambridge, U.K.

Pigott CD, Huntley JP (1981) Factors controlling the distribution of *Tillia cordata* at the northern limits of its geographical range III. Nature and causes of seed sterility. *New Phytologist* **87**, 817-839.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution* **43**, 223-225.

Silva-Montellano A, Eguiarte LE (2003) Geographic patterns in the reproductive ecology of *Agave lechuguilla* (Agavaceae) in the Chihuahuan desert. I. Floral characteristics, visitors, and fecundity. *American Journal of Botany* **90**, 377-387.

Stuessy T (1990) *Plant Taxonomy: The Systematic Evaluation of Comparative Data* Columbia University Press, New York.

Takhtajan A (1981) *Flowering plants: origin and dispersal* Bishen Singh Mahendra Pal Singh and Otto Koeltz Science Publishers, Koenigstein.

Talavera S (1987) Caryophyllaceae. In: *Flora vascular de Andalucía Occidental* (eds. Valdés B, *et al.*), pp. 206-276. Ketres editora, Barcelona.

Talavera S, Gibbs PE, Herrera J (1993) Reproductive biology of *Cistus ladanifer* (Cistaceae). *Plant Systematics and Evolution* **186**, 123-134.

Trabaud L (1995) Modalités de Germination des Cistes et des Pins Méditerranéens et Colonisation des Sites Perturbés. *Revue d'Écologie (Terre Vie)* **50**, 3-14.

Valbuena L, Alonso I, Tárrega R, Luis E (1992) Influencia del calor y del aclarado sobre la germinación de *Cistus laurifolius* y *Cistus ladanifer*. *Pirineos* **140**, 109-118.

Woodward FI, Williams BG (1987) Climate and plant distribution at global and local scales. *Vegetatio* **69**, 189-197.

Zar JH (1999) *Biostatistical analysis* Prentice Hall, Englewood Cliffs, New Jersey.

Appendix 1

Table of localities, geographical parameters, climatic characteristics and intra-specific level characteristics of the studied populations of *Cistus ladanifer*

Appendix 1. Sites, geographical parameters, climatic characteristics and infra-specific level characteristics of the studied populations of *C. ladanifer*. Populations analysed in the altitude study in bold.

CODE	LOCALITY	SUBSPECIES/ HAPLOTYPE*	LATITUDE	LONGITUDE	ALTITUDE (m a.s.l.)	T (°C)	T_h (°C)	PREC (mm)
Mon	Montpellier, Alsace, France	ladanifer	43° 36′ N	3°52′ W	185	13.9	22.2	710
Lar	Laroco, Orense, Spain	ladanifer/1	42° 21′ N	7° 10′ W	512	10.5	18.0	927
Zam	Peñausende, Zamora, Spain	ladanifer/1	41° 14′ N	5° 53′ W	960	13.2	23.6	443
Sal	Valdunciel, Salamanca, Spain	ladanifer/1	41° 09′ N	5° 41′ W	844	11.8	21.2	459
Ata	El Atazar, Madrid, Spain	ladanifer/1	40° 55′ N	3° 28′ W	1000	12.5	22.7	692
Cab	La Cabrera, Madrid, Spain	ladanifer/1	40° 52′ N	3° 37′ W	1000	12.5	22.7	692
Gua1	La Barranca, Madrid, Spain	ladanifer/1	40° 45′ N	4° 00′ W	1400	6.5	16.3	1170
Man	Manzanares el Real, Madrid, Spain	ladanifer/1	40° 43′ N	3° 52′ W	900	12.7	23.7	725
Esc	El Escorial, Madrid, Spain	ladanifer/1	40° 34′ N	4° 07′ W	900	13.4	24.2	637
Can	Tres Cantos, Madrid, Spain	ladanifer/1	40° 34′ N	3° 42′ W	700	12.7	23.7	725
Gua 2	El Pardo, Madrid, Spain	ladanifer/1	40° 30′ N	3° 45′ W	678	13.9	24.2	438
Boa	Boadilla del Monte, Madrid, Spain	ladanifer/1	40° 23′ N	3° 52′ W	650	12.9	24.6	404
Cha	Chapinería, Madrid, Spain	ladanifer/1	40° 22′ N	4° 12′ W	600	12.9	24.6	404
Jer	Jerte, Cáceres, Spain	ladanifer/1	40° 16′ N	6° 06′ W	868	14.4	23.8	1138
Lin	Linares, Jaén, Spain	ladanifer	38° 08′ N	3° 43′ W	350	17.6	28.3	541
Nav	Las Navas de la Concepción, Sevilla, Spain	ladanifer	37° 52′ N	5° 24′ W	350	14.5	22.9	813
Nor2	Sª Norte, Seville, Spain	ladanifer	37° 55′ N	5° 34′ W	803	14.5	22.9	813
Nor1	Sª Norte, Seville, Spain	ladanifer/1	37° 54′ N	5° 25′ W	322	14.5	22.9	813
Hig	Higuera de la Sierra, Huelva, Spain	ladanifer	37° 49′ N	6° 27′ W	600	14.5	24.6	1026
Peñ	Peñaflor, Sevilla, Spain	ladanifer	37° 45′ N	5° 22′ W	270	17.5	27.6	687
Val	Valverde del Camino, Huelva, Spain	ladanifer	37° 34′ N	6° 45′ W	250	19.2	28.3	782
Hin	Hinojos, Huelva, Spain	ladanifer/1	37° 17′ N	6° 22′ W	80	17.1	26.5	618
Alh2	Alhamilla mountain, Almería, Spain	ladanifer/6	36° 59′ N	2° 22′ W	1294	17.9	26.9	221
Isl	Isleta del Moro, Almería, Spain	ladanifer/6	36° 52′ N	2° 00′ W	370	17.3	25.9	268

Appendix 1. *(Continued)*

Alh1	Poyatos, Almería, Spain	ladanifer	36° 45′ N	2° 09′ W	240	17.3	25.9	268
Alg	Algar, Cádiz, Spain	ladanifer	36° 38′ N	5° 39′ W	480	17.7	25.9	728
Ber2	Sª Bermeja, Málaga, Spain	africanus/6	36° 34′ N	5° 15′ W	568	14.7	23.3	1189
Ber1	Sª Bermeja, Málaga, Spain	africanus	36° 32′ N	5° 10′ W	944	14.7	23.3	1189
Asi	Asilah, Tánger, Morocco	africanus/4	35° 46′ N	5° 55′ W	20	-	-	-
Chao	Chaouen, Chaouen, Morocco	africanus	35° 04′ N	5° 14′ W	570	17.4	31.9	944
Bab	Bab-Taza, Chaouen, Morocco	ladanifer/1	35° 04′ N	5° 10′ W	1050	14.9	32.3	1482
Dje	Djebel Bouhalia, Chaouen, Morocco	ladanifer/1	35° 04′ N	5° 10′ W	600	14.9	32.3	1482
Ben	Beni-Hadifa, Al Hoceima, Morocco	africanus/4	35° 01′ N	4° 09′ W	1065	15.2	30.6	900
Tar	Targhist, Al Hoceima, Morocco	africanus/4	34° 57′ N	4° 21′ W	1240	14.4	30.8	300
Ket	Ketama, Al Hoceima, Morocco	africanus/4	34° 53′ N	4° 35′ W	1400	11.4	26.9	900
Taf	Taforalt, Oudja, Morocco	africanus/7	34° 49′ N	2° 29′ W	874	-	-	-

* Guzmán, B. & Vargas, P., unpublished

T, mean annual temperature; T_h, mean temperature of the hottest month; PREC, mean annual precipitation

Climatic data from the Worldwide Bioclimatic Classification System (http://www.ucm.es/info/cif/data/indexc.htm)

8
Resumen y conclusiones

En los últimos 20 años el empleo de técnicas moleculares en el campo de la Sistemática y evolución ha contribuido enormemente a entender las relaciones filogenéticas entre los organismos en todos los niveles taxonómicos (veáse proyecto *Tree of Life*, http://www.tolweb.org/tree/). Dentro del grupo de las angiospermas, las clasificaciones tradicionales basadas en caracteres no moleculares (morfológicos, anatómicos, etc.) se han visto mayormente apoyadas por aquellas obtenidas del estudio de caracteres moleculares, aunque incluyen puntualizaciones sustanciales (Soltis *et al.* 2005). La existencia de un mayor número de datos, la baja ambigüedad en sus estados de carácter, la mejor identificación de homologías y la disponibilidad de múltiples marcadores neutrales son algunas de las ventajas que los datos moleculares ofrecen frente a los no moleculares (Graur & Li 1999). Por ello, hipótesis filogenéticas derivadas del análisis de secuencias de ADN son consideradas la base para describir patrones evolutivos, para después someter a test hipótesis evolutivas concretas (Chase *et al.* 2000).

1. Resultados más notables de la memoria doctoral

El conjunto de resultados presentados a lo largo de esta memoria doctoral (capítulos 2 y 3) sugiere una clasificación de la familia Cistáceas diferente para algunos géneros a la derivada del estudio de caracteres morfológicos sugerida por autores previos (Dunal 1824; Spach 1836; Willkomm 1856; Grosser 1903). Las reconstrucciones de estados de carácter (capítulo 2) muestran un alto nivel de homoplasia en algunos de los caracteres empleados en la clasificación de las Cistáceas (hábito, disposición y forma de inserción de las hojas, color de pétalo). Por el contrario, un buen número de caracteres morfológicos (número de sépalos, número de carpelos, forma del embrión, tipo de polen, etc.) han permitido una correcta agrupación de poblaciones y especies en grupos naturales (o casi). Este hecho hace que los caracteres morfológicos sigan siendo de gran valor taxonómico, si bien la adquisición de diferentes rasgos evolutivos se va determinando gracias a los análisis filogenéticos basados en secuencias nucleotídicas. Un ejemplo claro es el color del pétalo en el complejo *Cistus-Halimium*. La presunción de que los dos colores presentes en las especies de *Cistus* (blanco y rosa-púrpura) podrían

servir para definir dos grupos naturales (Dunal 1824; Spach 1836; Willkomm 1856; Grosser 1903; Dansereau 1939), a pesar de su aparente variabilidad en otros grupos de angiospermas, se ha confirmado con nuestros resultados de dos linajes independientes. En concreto las 11 especies de flor blanca forman un grupo monofilético (a partir de este momento le denominaremos linaje de jaras de flor blanca) frente al grupo monofilético de 9 especies de flor púrpura (linaje de jaras de flor púrpura), aunque con una única excepción. El conjunto de hipótesis filogenéticas derivadas del análisis de distintas regiones del ADN (capítulos 2, 3, 4, 5 y 6) han mostrado claramente que *Cistus parviflorus* (especie de flor rosa) posee un mayor grado de parentesco con las especies de flor blanca que con las especies de flor púrpura.

Por otro lado, los resultados presentados manifiestan la existencia de una estrecha cohesión entre las especies de *Cistus* y *Halimium* (capítulos 2, 3, 4, 5 y 6), ya sugerida por diversos autores previamente (Dansereau 1939), que nos ha llevado a dejar de hablar de ambos géneros como unidades individuales para referirnos a ellos como el complejo *Cistus-Halimium*. Las especies de ambos grupos comparten importantes características, entre las que destacamos: números cariológicos idénticos (Dansereau 1939), similitud en la morfología de los granos de polen (Saenz 1979; Ukraintseva 1993), posibilidad de hibridación dando lugar al híbrido intergenérico *Halimiocistus*. Diversos autores han propuesto la unión de ambos grupos en un único género (Löve & Kjellqvist 1964; Demoly 2006), sin embargo como ya apuntó Dansereau (1939), a pesar de que las especies de ambos grupos han debido sufrir una evolución "paralela" cada grupo posee características propias que dificultan su unión.

Los estudios biogeográficos y de datación molecular realizados (capítulos 3, 4 y 6) establecen un origen y posterior diversificación del complejo *Cistus-Halimium* paralelos al origen y establecimiento del clima Mediterráneo en Europa y África (Suc *et al.* 1995). Especiación asociada a radiación parece ser el proceso más común responsable del origen de especies dentro del complejo *Cistus-Halimium*. Este hecho no es sorprendente cuando numerosos estudios filogenéticos están poniendo de manifiesto que una gran cantidad de la diversidad biológica se ha originado durante episodios de radiación en

regiones de máxima biodiversidad (Myers *et al.* 2000). Los distintos ambientes a los que dio lugar el establecimiento del clima Mediterráneo promovieron una rápida diversificación en el linaje de *Cistus* de flor blanca (capítulo 4). Numerosos estudios han puesto de manifiesto el importante papel que las características foliares de tipo morfológico y fisiológico manifiestan en la colonización de diferentes ambientes (Givnish 1979, 1987; Ackerly *et al.* 2002). El alto número de especies incluidas en el linaje *C. libanotis* (10 especies) respecto a su grupo hermano (2), el bajo porcentaje de divergencia nucleotídica en las secuencias encontrada entre especies morfológicamente muy diferentes (aún empleando una combinación de seis regiones distintas del ADN nuclear (2) y plastidial (4)) y el alto grado de variabilidad en la morfología foliar encontrado dentro del linaje (comparado no solo con su grupo hermano (4 tipos vs. 1) sino con el resto de linajes de la familia) pone de manifiesto no sólo uno de los escasos ejemplos de radiación adaptativa documentado en el continente sino el primero en un grupo de amplia distribución (región Mediterránea).

Por el contrario, los casos de radiación adaptativa en sistemas insulares son muy abundantes en la literatura (Baldwin & Robichaux 1995; Böhle *et al.* 1996; Givnish *et al.* 1996; Kim *et al.* 1996; Francisco-Ortega *et al.* 1997). La mayor diversidad de hábitats y el aislamiento de las islas oceánicas permiten, con respecto a los sistemas continentales, la existencia de una menor competencia y una mayor cantidad de nichos ecológicos vacíos que promueven un alto grado de proliferación de especies (Grant 1998). Sin embargo, las oportunidades ecológicas en un periodo corto de especiación en el continente suelen estar tan subestimadas que nos resulta sorprendente encontrar grupos continentales de amplia distribución con igual o mayor grado de diversificación que su respectivo grupo hermano insular. En el capítulo 5 se argumenta que una evolución de similares dimensiones ha ocurrido dentro del linaje de jaras de flor púrpura para dar origen primero a *C. crispus*, y después al linaje de jaras Canarias y su grupo hermano continental (3 especies, i. e. resto de jaras de flor púrpura). En concreto, una diferenciación sincrónica se refleja no sólo en el porcentaje de divergencia de secuencias (0,21% vs. 0,27%) y en la edad en que ambos linajes empezaron a divergir (0,33 ± 0,14

vs. 0,66 ± 0,15 millones de años), sino también en el número similar de especies (5 vs. 3), haplotipos (7 vs. 6) y linajes de haplotipos (4 vs. 4) encontrados en ambos grupos. ¿Significan estos resultados que el linaje de jaras Canarias ha experimentado una radiación más lenta de lo habitual para un linaje insular? Nuestros resultados apuntan a que la corta edad de este linaje (330.000 años) hace que nos encontremos ante una radiación no adaptativa incipiente en parte enmascarada por la radiación sufrida en el linaje hermano continental debido a su distribución en otro *hotspot* como es la región Mediterránea.

El estudio de las relaciones existentes entre la genealogía y la geografía de linajes dentro de una misma especie (Avise 2000), o filogeografía, debe ser realizado a través de marcadores moleculares. En plantas, el ADN plastidial, por sus características de estabilidad estructural, haploidía, herencia uniparental y variación estructurada geográficamente (Soltis *et al.* 1997; Zhang *et al.* 2005), se ha convertido en una fuente de datos muy útil en la inferencia genealógica a nivel intraespecífico. Avise (1987) agrupó en cinco categorías todos los posibles resultados obtenidos al superponer las filogenias intraespecíficas en los mapas de distribución de las especies y estableció que la más común de todas ellas era aquella que presentaba discontinuidades filogenéticas debidas a una separación espacial en el área de distribución. El Estrecho de Gibraltar (discontinuidad espacial del área de distribución de la jara pringosa) ha sido considerado desde siempre una barrera al flujo génico entre poblaciones de ambos lados. Este hecho, junto a la carencia de estructuras facilitadoras de la dispersión en las semillas y frutos de *C. ladanifer*, hacían presuponer la existencia de una estructura genética diferente en las poblaciones europeas y africanas. El estudio filogeográfico (capítulo 6) reveló una estructura genética en parte uniforme a ambos lados del Estrecho (el haplotipo 1 se encontró en poblaciones de *C. ladanifer* subespecie *ladanifer* situadas en Europa y norte de África) fácilmente explicable a través de un proceso de vicarianza. Sin embargo, la edad de divergencia estimada para *C. ladanifer* (1,73 – 0,73 millones de años) y la edad de reapertura del Estrecho de Gibraltar (5 millones de años, Duggen *et al.* 2003) nos permiten decir no sólo que la distribución disyunta de la especie

se debe a múltiples eventos de dispersión a larga distancia (al menos dos), que ponen de manifiesto el escaso papel del estrecho de Gibraltar como barrera en la dispersión de la jara pringosa, si no que ha habido una activa dispersión a pesar de que sus frutos y semillas no tienen adaptaciones para migraciones a larga distancia. Todo ello, junto con la estrecha relación ecológica entre la península Ibérica y el norte de África, sugiere que las condiciones ambientales han debido ser decisivas en la colonización de *Cistus* a través del Estrecho de Gibraltar y a lo largo de todo Mediterráneo. El escaso papel del Estrecho de Gibraltar como barrera geográfica queda de nuevo patente en el segundo estudio microevolutivo realizado de la especie (capítulo 7), pues tampoco se observan patrones geográficos derivados de él en la variabilidad del número de valvas y semillas del fruto de la jara pringosa. De hecho este estudio puso de manifiesto que la variabilidad en el número de valvas y semillas del fruto no se debía a variables geográficas, taxonómicas o filogeográficas sino a plasticidad fenotípica.

2. Evaluación de procesos macro- y microevolutivos en el complejo *Cistus-Halimium*

Como ya apuntamos en la introducción de la presente memoria, por macroevolución se entiende la diferenciación de organismos por encima del nivel de especie mientas que la microevolución atiende a la diferenciación ocurrida en niveles inferiores al de especie (Stearns & Hoekstra 2001). Sin embargo, ¿debemos entender la macroevolución como un concepto puramente descriptivo surgido de la extrapolación de procesos evolutivos ocurridos en niveles infraespecíficos (microevolución) o debemos referirnos a ella en términos mecanicistas entendiendo que los cambios ocurridos en niveles supraespecíficos son causados por mecanismos diferentes a los que operan en niveles inferiores? La introducción de una escala espacio-temporal (fósiles, eventos geológicos, relojes moleculares) en los estudios evolutivos actuales está estableciendo límites fundamentales en la diversificación biológica a la hora de testar la hipótesis macroevolutiva.

A lo largo de la historia se han realizado estimaciones del tiempo que requiere el proceso de especiación. Así por ejemplo, Levin & Wilson (1976) establecieron que la tasa de especiación en grupos arbustivos era de 0,28 especies por millón de años. Esta tasa supone que 3,57 millones de años son necesarios para que se origine una nueva especie. Esto significa que el proceso de especiación supone un periodo de tiempo tal que cambios en el fenotipo y genotipo podrían explicarse mediante principios básicos de genética de poblaciones, y por tanto, mediante la acumulación de eventos microevolutivos. Sin embargo, trabajos evolutivos actuales (p.e. Kay *et al.* 2005; Hughes & Eastwood 2006), así como los resultados presentados en esta memoria (capítulos 4 y 5), están poniendo de manifiesto tasas de especiación muy superiores que no dan cabida a dicha acumulación como mecanismo generador de especies. Los análisis de datación molecular presentados en esta memoria (capítulos 4, 5 y 6) establecen un rango de edad para el origen del complejo *Cistus-Halimium* comprendido entre 3,88-1,66 millones de años. Es decir que, sin tener en cuenta las extinciones ocurridas en los distintos linajes, 29 especies han sido originadas prácticamente en el mismo periodo de tiempo (o inferior) en el que Levin & Wilson (1976) establecieron el origen de una única especie.

La relación entre diferenciación morfológica y corto lapso de tiempo para ser generada dentro de los dos linajes de jaras refleja notables cambios fenotípicos que se pueden vincular a procesos de especiación rápida. Un ejemplo sería el aumento en la longitud del estilo ocurrido únicamente en el linaje de endemismos canarios. La reconstrucción de la evolución de este carácter en las Cistáceas (Fig. 5A, capítulo 3) y la estimación de las edades de divergencia de los distintos linajes de jaras de flor púrpura (capítulo 5) nos permiten sugerir que en 900,000 años un ancestro de estilo con longitud similar a la de los estambres y posiblemente autoincompatible, tuvo que colonizar las islas, desarrollar un sistema de polinización autocompatible (lo que favorecería el establecimiento de poblaciones a partir de un único individuo) y posteriormente desarrollar un estilo de longitud superior a la de los estambres para propiciar una posterior separación de los sexos de una misma flor. Una especiación rápida por

aislamiento geográfico se produjo (5 especies en 0,33 ± 0,14 millones de años), pero manteniendo este carácter único en el género.

Así mismo, la radiación adaptativa podría ser entendida como un proceso macroevolutivo que actuaría favoreciendo a los linajes más lábiles y sería el mecanismo responsable de la alta tasa de especiación (2,13-3,49 especies por millón de años) encontrada para el linaje de *C. libanotis* (capítulo 4). Las notables diferencias morfológicas encontradas incluso entre especies hermanas, sería otro ejemplo que apoyaría nuestra hipótesis de que en el grupo de las jaras acumulaciones de cambios paulatinos ha contribuido, pero no parece haber sido el único mecanismo necesario para dar lugar al cambio evolutivo observado.

3. Mecanismos macroevolutivos en plantas modelo (género *Antirrhinum*)

Tres mecanismos macroevolutivos han sido descritos en plantas modelo, dada la posibilidad de un estudio génico detallado que implica detección de grandes cambios evolutivos en un periodo corto de tiempo. En concreto, en la tribu Antirrhineae se ha inferido la importancia de tres mecanismos:

1. Mutagénesis en la secuencia nucleotídica. Cambios mutacionales en el patrón de expresión génica de los genes *CYCLOIDEA* y *DICHOTOMA* producen importantes cambios en la forma floral, de manera que la flor personada característica de *Antirrhinum* puede convertirse en una flor (casi) actinomorfa en el género *Mohavea* (Hileman *et al.* 2003) (Fig. 1). Ello se traduce, obviamente, en importantes consecuencias en la biología evolutiva del grupo ya que los polinizadores de ambas flores pasan a ser completamente diferentes.

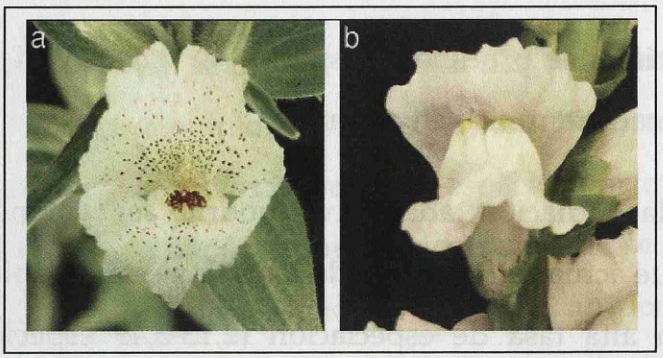

Fig. 1. (a) Forma floral de *Mohavea confertiflora* (simetría radial). (b) Forma floral de *Antirrhimum majus* (simetría bilateral) (Hileman *et al.* 2003).

2. Mutagénesis proteíca en el ADN (epigenética). Por primera vez se demuestra que cambios puntuales en las proteínas del ADN (no en la secuencia nucleotídica) en *Linaria vulgaris* por medio de una metilación puntual de tipo proteico (variación epigenética) en el gen *Lcyc*, que es homólogo de CYCLOIDEA, supone un cambio sobresaliente al trasformar una flor zigomorfa propia de *Linaria vulgaris* en una flor actinomorfa pelórica, que ya Linneo describiera en su tiempo (Fig. 2) (Cubas *et al.* 1999).

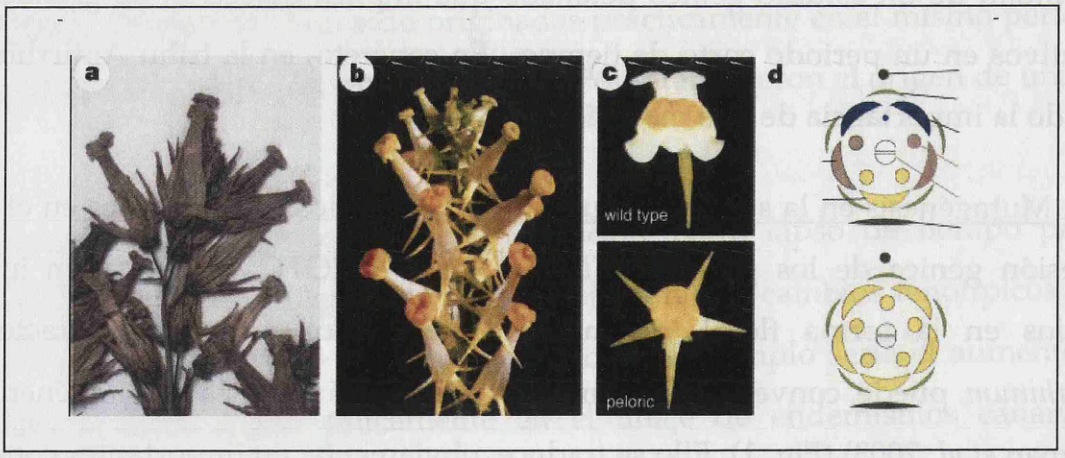

Fig. 2. Inflorescencia de *Linaria vulgaris* de flores actinomorfas pelóricas de un espécimen de herbario recolectado por Linneo (a) y un espécimen vivo (b). Vista del tipo silvestre (arriba) frente al mutante pelórico (abajo) (c). Diagrama floral del tipo silvestre (arriba) frente al mutante pelórico (abajo) mostrando la posiciones relativas de los distintos órganos (d) (Cubas *et al.* 1999).

3. Aloploidización. Poliploidía asociada a hibridación (alopoliploidía) parece haber sido el mecanismo más efectivo en el proceso macroevolutivo. De hecho, en una sola generación se puede crear una especie nueva que contenga dos genomas completos

diferentes que se han beneficiado de la acumulación de cambios en largos periodos de tiempo. La tribu Antirrhineae no parece haber estado exenta de este proceso, tal y como se deduce de estudios filogenéticos preliminares entre la comparación del genoma plastidial frente al nuclear (Vargas *et al.* 2004). La poliploidía es una fuerza tan importante en la evolución de las plantas que llega a ser el fenómeno más frecuente en el proceso de especiación simpátrica (Otto & Whitton 2000). No sólo muchos de los géneros de angiospermas contienen un alto porcentaje de poliploides sino que se considera que la mayoría de las angiospermas presentan genomas "paleopoliploides". Por tanto, procesos macroevolutivos por poliploidía asociada a hibridación (alopoliploidía) son muy frecuentes en angiospermas (Soltis *et al.* 2004; Wendel & Doyle 2005).

4. Predominancia de mecanismos macro- o microevolutivos en el complejo *Cistus-Halimium*

La presente memoria doctoral no ha conseguido discernir entre mecanismos propios de carácter macro- o microevolutivos. De hecho el planteamiento del estudio no pretendía profundizar en estos mecanismos. Sin embargo, el conjunto de datos acumulados a lo largo del estudio histórico de este complejo nos permite postular si ha habido procesos evolutivos más importantes que otros.

En el caso de las Cistáceas aneuploidía y poliploidía se barajaban como dos procesos de gran importancia en la evolución de la familia. De hecho a pesar de que el número cromosómico gametofítico está bastante conservado tanto a nivel genérico como en niveles inferiores (capítulo 2), hay casos como el de *Tuberaria* sección *Scorpioides* donde poblaciones tetraploides (n = 12), hexaploides (n = 18) y octoploides (n = 24) (aunque no diploides) pueden encontrarse en la misma especie (Gallego & Aparicio 1993). Sin embargo, en el caso del complejo *Cistus-Halimium* hablar de

poliploidía como proceso macroevolutivo no tiene sentido cuando todas sus especies son homoploides ($n = 9$).

La hibridación como fenómeno común en las Cistáceas es conocida desde Linneo, de hecho la gran cantidad de híbridos producidos por el cruce de dos o más especies (Gard 1910, 1912, 1914; Demoly 1996) motivó su frase: *Cistorum Historia, maxime omnium obscura ob varietum copia*. Sin embargo, el importante papel otorgado a la hibridación como agente generador de especies dentro del complejo *Cistus-Halimium* (Dansereau 1939) queda poco patente a la luz de los resultados presentados, si bien es cierto que el estudio de secuencias de las regiones plastidiales e ITS (Internal Transcriber Spacers) del ADN ribosómico mostró indicios de procesos de hibridación interespecífica recientes en el grupo de jaras de flor púrpura (capítulo 3).

Por todo ello concluimos que la célebre frase de Darwin (1859), *natura non facit saltum*, sigue siendo válida al contemplar el cambio evolutivo predominante en plantas, si bien hay otros procesos implicados. De hecho, nuestros resultados apuntan a que un mecanismo "a saltos" se ajustaría a diversos cambios observados. Futuros estudios de mutaciones puntuales en la secuencia de ADN (mutagénesis nucleotídica) o en las proteínas del ADN (mutagénesis epigenética) serían necesarios para valorar el papel de los notables cambios morfológicos observados en el complejo *Cistus-Halimium*. Nuestro estudio demuestra que un tiempo limitado (muy inferior al tiempo estimado de especiación en angiospermas; véase Levin & Wilson 1976; Eriksson & Bremer 1992) produce significativos cambios morfológicos. En concreto, la longitud del estilo (única en las jaras canarias), un numero de valvas variable entre 5-12 (en *C. ladanifer*), hojas atípicas en la familia (especialmente las de *C. populifolius* y *C. ladanifer*) se han originado de manera súbita (Tabla 1).

Tabla 1. Variabilidad morfológica de las especies de jaras. Datos tomados de diversas floras, Dansereau (1939), Demoly and Montserrat (1993) y observaciones personales

	Forma de la hoja	Color de la flor	Nº de sépalos	Longitud del estilo (mm)	Número de valvas
C. albanicus	Elíptica	Blanco	5	<0.5	5
C. albidus	Oblonga-elíptica	Púrpura	5	2.5-5	5
C. chinamadensis	Lancelada-elíptica	Púrpura	5	7-12	5
C. clusii	Linear	Blanco	3	1-2	5
C. creticus	Oblonga-elíptica	Púrpura	5	2-5	5
C. crispus	Oblonga-elíptica	Púrpura	5	2-5	5
C. heterophyllus	Lanceolada-elíptica	Púrpura	5	2-5	5
C. horrens	Oval	Púrpura	5	6-10	5
C. ladanifer	Linear-lanceolada	Blanco	3	0	5-12
C. laurifolius	Ovada-lanceolada	Blanco	3	<0.5	5
C. libanotis	Linear	Blanco	3	1	5
C. monspeliensis	Linear-lanceolada	Blanco	5	0.5	5
C. munbyi	Linear	Blanco	3	1-2	5
C. ochreatus	Ovada-oblonga	Púrpura	5	6-10	5
C. osbeckiifolius	Lanceolada-elíptica	Púrpura	5	6-10	5
C. parviflorus	Ovada	Rosa	5	0	5
C. populifolius	Ovada-lanceolada	Blanco	5	0	5
C. pouzolzii	Lanceolada-elíptica	Blanco	5	2-5	5
C. psilosepalus	Lanceolada-elíptica	Blanco	5	0.5	5
C. salviifolius	Ovada	Blanco	5	0	5
C. symphytifolius	Lanceolada-ovada	Púrpura	5	10-20	5

La biología evolutiva del desarrollo (Evo-Devo) está poniendo de manifiesto que las grandes diferencias morfológicas existentes entre los organismos de los distintos filos no aparecen siempre reflejadas a nivel génico. Así por ejemplo, dentro de las gramíneas la macroevolución se relaciona con cambios en la posición de los programas de desarrollo (evolución heterocrónica), posiblemente por la vía de la expresión ectópica de los genes (Kellogg 2002). De esta forma, todos los organismos presentarían un "kit" de genes común que hace que la diversidad emerja no sólo del contenido si no de las distintas formas en que es empleado (Carrol 2005). Así pues la diversidad en animales, plantas y demás organismos sería el producto de variaciones en planes corporales ancestrales más que de novedades evolutivas. De hecho, la genómica aporta cada vez más pruebas de que una vez conseguidos unos genes particulares de desarrollo, éstos pueden mantenerse silenciados a lo largo del tiempo, para después volverse a "reciclar" cuando encuentran la posibilidad de desarrollarse ante las condiciones impuestas por la selección natural.

Conclusiones:

1. Los resultados obtenidos en la presente memoria doctoral confirman la formación de cinco grupos naturales congruentes con cinco (*Crocanthemum, Fumana, Helianthemum, Hudsonia, Tuberaria*) de los siete géneros de la familia Cistaceae. *Cistus* y *Halimium* no son independientes y forman un grupo natural.

2. Se reconocen los géneros *Helianthemum* y *Crocanthemum* como entidades taxonómicas independientes, aunque estrechamente relacionadas.

3. Los análisis filogenéticos y las reconstrucciones evolutivas de estados de carácter confirman la hipótesis de Nandi basada en el desarrollo floral sobre el estado ancestral de *Fumana*, debido a que es el género hermano al resto de géneros de Cistáceas.

4. Las especies de *Halimium* y *Cistus*, íntimamente ligadas, forman un grupo natural, reconociéndose, tal y como hicieron otros autores anteriormente sobre la base de caracteres morfológicos, una relación particularmente estrecha entre *Halimium umbellatum* y el linaje formado por las especies de *Cistus* de flor blanca (más *Cistus parviflorus*).

5. La división tradicional de las especies de *Cistus* en dos grupos según el color de los pétalos (púrpura, blanco) es básicamente congruente con los resultados filogenéticos obtenidos al formar las especies de flor blanca (más *C. parviflorus*) un linaje independiente frente al de las especies de flor púrpura. Los tres subgéneros propuestos en los últimos tratamientos taxonómicos han resultado ser no monofiléticos.

6. Tanto el número de especies de *Cistus* (14 de 21) y *Halimium* (8 de 12) como las relaciones de parentesco entre las mismas sugieren que el oeste del mediterráneo es un centro de diversificación de ambos géneros.

7. Aunque la divergencia entre las familias Dipterocarpaceae y Cistaceae tuvo lugar en el Eoceno-Oligoceno y la diversificación de las Cistáceas ya había ocurrido durante el Mioceno, no es hasta finales del Plioceno cuando se produce una rápida y notable diversificación dentro del complejo *Cistus-Halimium* coincidiendo con el establecimiento del clima Mediterráneo.

8. El linaje de jaras de flor blanca ha experimentado una radiación adaptativa expresada en variaciones en caracteres foliares que han permitido el rápido éxito de las especies en distintos ambientes ecológicos desde el Pleistoceno. Se trata pues, según nuestro conocimiento, del primer caso de radiación adaptativa documentado para plantas mediterráneas.

9. El patrón general de una mayor tasa de diversificación en grupos insulares que en continentales no es apoyado por nuestros resultados filogenéticos, filogeográficos y de datación molecular en relación a las especies de *Cistus* de flor púrpura canarias (5) y continentales (3). Los resultados similares obtenidos al comparar ambos grupos son empleados para describir dos historias evolutivas sincrónicas de los linajes canario y continental al enfrentarse ante dos puntos calientes de diversidad: las regiones Macaronésica y Mediterránea.

10. La distribución de los haplotipos del linaje Canario muestra que una radiación por aislamiento geográfico, más que ecológico, podría explicar el origen de endemismos de jaras. La llegada a las islas de un único antepasado en tiempos recientes (< 800.000 años) podría explicar la limitada diferenciación de *Cistus* en Canarias.

11. El hecho de que el origen de *Cistus ladanifer* sea posterior a la reapertura del Estrecho de Gibraltar nos permite explicar, por medio de análisis filogenéticos y filogeográficos, que la distribución disyunta de la especie es debida a múltiples eventos de dispersión a larga distancia desde el continente africano al europeo. A pesar de sus escasos mecanismos de dispersión, la preferencia de las jaras por hábitats xéricos ha sido decisiva para una exitosa colonización tras el inicio del clima

mediterráneo (c. 3.2 Ma), cuando las especies de *Cistus* empezaron a formar parte del elemento dominante en el matorral mediterráneo.

12. La gran variabilidad presentada por *Cistus ladanifer* en el número de divisiones del fruto podría ser explicada, en parte, como una respuesta plástica ante determinados factores ecogeográficos.

13. El gradualismo filético, eje principal de la teoría sintética de la evolución, no parece ser el único fenómeno responsable de la diferenciación observada en *Cistus*. Como quiera que la poliploidía no parece haber jugado un papel relevante en la diversificación del complejo *Cistus-Halimium*, los resultados de la presente memoria doctoral sugieren que otros mecanismos macroevolutivos están implicados en la aparición de caracteres claves. La brusca adquisición de nuevas formas ha debido de ser desencadenada por las nuevas condiciones ecológicas proporcionadas por el clima mediterráneo. Además, la reaparición rápida de caracteres a partir de la expresión de genes silenciados ya existentes, que vuelven a ser apropiados para enfrentarse a determinadas condiciones ecológicas, podría ser una constante en la evolución de *Cistus*.

Conclusions:

1. Our results confirm naturalness of five (*Crocanthemum, Fumana, Helianthemum, Hudsonia, Tuberaria*) of the seven Cistaceae genera. *Cistus* and *Halimium* are not independent and form a natural group.

2. *Helianthemum* and *Crocanthemum* are recognised as independent, but closely-related, taxonomic entities.

3. Phylogenetic analysis and historical inferences of character state evolution strongly support Nandi´s hypothesis based on flower development given the sister-group relationship of *Fumana* with the rest of Cistaceae genera.

4. *Halimium* and *Cistus* species form a cohesive natural group. In particular, a very close relationship between *Halimium umbellatum* and the white-flowered *Cistus* species (plus *C. parviflorus*) is observed, as previous authors stated using morphological characters.

5. Traditional classification of *Cistus* species in two groups based on petal colors (purple, white) is primarily congruent with our phylogenetic results, in which white-flowered species (plus *Cistus parviflorus*) form an independent linage to the purple-flowered lineage. The three subgenera proposed in the last taxonomic treatments are however not monophyletic.

6. Both the number of species of *Cistus* (14 of 21) and *Halimium* (8 of 12) and topological relationships of lineages suggest that the western Mediterranean is a center of diversity for the two genera.

7. Although the Dipterocarpaceae and Cistaceae divergence had taken place around the Eocene-Oligocene boundary and Cistaceae diversification in the Miocene, the fast and remarkable diversification in the *Cistus-Halimium* complex occurred at the end of the Pliocene coinciding with the establishment of the Mediterranean climate.

8. The white-flowered *Cistus* lineage underwent an adaptive radiation. Shifts in leaf key innovations have been crucial to exploit the different ecological conditions of the Mediterranean habitats. Adaptive radiation of Mediterranean species is documented for the first time, as far as we know.

9. The general pattern of higher diversification rates in island with respect to mainland groups is not supported by our phylogenetic, phylogeographical and molecular clock results, regarding to the Canarian (5 species) and continental (3 species) species of purple-flowered *Cistus*. Our comparable results between the two groups are interpreted as strong evidence of two synchronous evolutionary histories of the Canarian and mainland lineages facing two hotspots of plant diversity: the Macaronesian and Mediterranean regions.

10. Haplotype distribution of the Canarian lineage shows a geographical pattern of differentiation, rather than ecological isolation. The arrival of a single introduction in recent times (< 800.000 years) may be responsible for limited differentiation in the Canary Islands.

11. The origin of *Cistus ladanifer* postdating the opening of the Strait of Gibraltar, as inferred by phylogenetic and phylogeographical analyses, allows explaining the disjunct distribution area of the species by means of multiple long-distance dispersal events from Africa to Europe. Despite limited dispersal abilities, preference to xeric habitats has been decisive to successful colonization after the advent of the Mediterranean climate (c. 3.2 Ma), when *Cistus* species early became part of the dominant element in the Mediterranean scrub.

12. A plastic response to particular ecogeographical factors accounts, in part, for a high level of variation observed in the number of fruit divisions of *Cistus ladanifer*.

13. The phyletic gradualism, main axis of the synthetic theory of evolution, does not appear to be the only phenomenon responsible of *Cistus* differentiation. Given that polyploidy has not been described in the *Cistus-Halimium* complex, our results

suggest that other macroevolutionary mechanisms are involved in rapid acquisition of key innovations. Sudden occurrence of new morphologies may have been triggered by new ecological conditions offered by the Mediterranean climate. Additionally, co-option of pre-existing genetic characteristics may facilitate rapid expression of previously silencing genes into characters newly suitable for particular ecological conditions in the course of *Cistus* evolution.

Referencias bibliográficas

Ackerly DD, Knight CA, Weiss SB, Barton K, Starmer KP (2002) Leaf size, specific leaf area and microhabitat distribution of chaparral woody plants: contrasting patterns in species level and community level analyses. *Oecologia* **130**, 449-457.

Avise JC (1987) Intraspecific phylogeography: the Mitochondrial DNA bridge between population genetics and systematics. **18**, 489-522.

Avise JC (2000) *Phylogeography: the history and formation of species* Harvard University Press, Cambridge.

Baldwin BG, Robichaux RH (1995) Historical biogeography and ecology of the Hawaiian silverswoord alliance (Asteraceae): new molecular phylogenetic perspectives. In: *Hawaiian biogeography: evolution on a hotspot archipelago* (eds. Wagner WL, Funk VA), pp. 257-287. Smithsonian Institution Press, Washington, D.C.

Böhle U-R, Hilger HH, Martin WF (1996) Island colonization and evolution of the insular woody habit in *Echium* L. (Boraginaceae). **93**, 11740-11745.

Carrol SB (2005) The origins of form. *Natural History* **11**, 58-63.

Cubas P, Vincent C, Coen E (1999) An epigenetic mutation responsible for natural variation in floral symmetry. *Nature* **401**, 157-161.

Chase MW, Fay MF, Savolainen V (2000) Higher-level classification in the angiosperms: new insights from the perspective of DNA sequence data. *Taxon* **49**, 685-704.

Dansereau P (1939) Monographie du genre *Cistus*. *Boissiera* **4**, 1-90.

Darwin C (1859) *The Origin of Species by Means of Natural Selection*, London.

Demoly JP (1996) Les hybrides binaires rares du genre *Cistus* L. (*Cistaceae*). *Anales Jardin Botanico de Madrid* **54**, 241-254.

Demoly JP (2006) Notes taxonomiques, chorologiques et nouveautés nomenclaturales pour le genre *Cistus* L. élargi, incluant *Halimium* (Dunal) Spach (Cistaceae). *Acta Botanica Gallica* **155**, 309-323.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Duggen S, Hoernle K, van den Bogaard P, Rüpke L, Morgan JP (2003) Deep roots on the Messinian salinity crisis. *Nature* **422**, 602-606.

Dunal (1824) Cistineae in De Candolle. A. P. *Prodromus Systematis Naturalis Regni Vegetabilis* **I**, 263-286.

Eriksson O, Bremer B (1992) Pollination systems, dispersal modes, life forms, and diversification rates in angiosmperm families. *Evolution* **46**, 258-266.

Francisco-Ortega J, Santos-Guerra A, Hines A, Jansen RK (1997) Molecular evidence for Mediterranean origin of the Macaronesian endemic genus *Argyranthemum* (Asteraceae). *American Journal of Botany* **84**, 1595-1613.

Gallego MJ, Aparicio A (1993) Karyological study in the genus *Tuberaria* sect. *Scorpioides* (Cistaceae): taxonomic and evolutionary inferences. *Plant Systematic and Evolution* **184**, 11-25.

Gard M (1910) Recherches sur les hybrides artificiels des Cistes, obtenus par M. Ed. Bornet. Premier mémoire: Notes inédites et résultats expérimentaux. *Annales des Sciences Naturelles, Botanique* **9**, 71-115.

Gard M (1912) Recherches sur les hybrides artificiels de Cistes, obtenus par M. Ed. Bornet. Deuxième mémoire: Les espèces et les hybrides binaires. *Beihefte zum Botanischen Centralblatt* **2**, 306-394.

Gard M (1914) Recherches sur les hybrides artificiels de Cistes, obtenus par M. Ed. Bornet. Troisième mémoire: Les hybrides dérivés et les hybrides complexes. *Beihefte zum Botanischen Centralblatt* **2**, 306-394.

Givnish TJ (1979) On the adaptive significance of leaf form. In: *Topics in plant population biology* (eds. Solbrig OT, *et al.*), pp. 375-407. Columbia University Press, New York.

Givnish TJ (1987) Comparative studies of leaf form: assessing the relative roles of selective pressures and phylogenetic constraints. *New Phytologist* **106 (Suppl.)**, 131-160.

Givnish TJ, Knox E, Patterson TB, *et al.* (1996) The Hawaiian lobelioids are monophyletic and underwent a rapid initial radiation roughly 15 million years ago. *American Journal of Botany (suppl.)* **83**, 159.

Grant PR (1998) Epilogue and questions. In: *Evolution on islands* (ed. Grant PR), pp. 305-319. Oxford University Press, New York.

Graur D, Li WH (1999) *Fundamentals of Molecular Evolution* Sinauer Associates Inc., Sunderland, Massachussetts.

Grosser W (1903) Cistaceae. In: *Dans Pflanzenreich* (ed. Engler A), p. 161, Berlin.

Hileman LC, Kramer EM, Baum DA (2003) Differential regulation of symmetry genes and the evolution of floral morphologies. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12814-12819.

Hughes C, Eastwood R (2006) Island radiation on a continental scale: exceptional rates of plant diversification after uplift of the Andes. *Proceedings of the National Academy of Sciences* **103**, 10334-10339.

Kay KM, Reeves PA, Olmstead RG, Schemske DW (2005) Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. *american Journal of Botany* **92**, 1899-1910.

Kellogg EA (2002) Are macroevolution and microevolution qualitatively different? evidence from Poaceae and other families. In: *Developmental genetics and plant evolution* (eds. Cronck QCB, *et al.*), pp. 70-84. Taylor & Francis, London.

Kim S-C, Crawford DJ, Francisco-Ortega J, Santos-Guerra A (1996) A common origin for woody *Sonchus* and five related genera in the Macaronesian islands: evidence for extensive radiation. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 7743-7748.

Levin DA, Wilson AC (1976) Rates of evolution in seed plants: net increase in diversity of chromosome numbers and species numbers through time. *Proceedings of the National Academy of Sciences* **73**, 2086-2090.

Löve A, Kjellqvist E (1964) Chromosome numbers of some Iberian Cistaceae. *Portugaliae Acta Biologica, serie A: morfologia, fisiologia, genetica y biologia general* **8**, 69-80.

Myers N, Mittermeier RA, Mittermeier CG, Fonsaeca GAB, Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature* **403**, 853-858.

Otto SP, Whitton J (2000) Poliploidy incidence and evolution. *Annual Review of Genetics* **34**, 401-437.

Saenz C (1979) Pollen morphology of Spanish Cistaceae. *Grana* **18**, 91-98.

Soltis DE, Gitzendanner MA, Strenge DD, Soltis PS (1997) Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution* **206**, 353-373.

Soltis DE, Soltis PS, Endress PK, Chase MW (2005) *Phylogeny and evolution of angiosperms* Sinauer Associates, Inc., Sunderland, Massachussetts.

Soltis DE, Soltis PS, Tate JA (2004) Advances in the study of polyploidy since plant speciation. *New Phytologist* **161**, 173-191.

Spach E (1836) Conspectus monographiae Cistacearum. *Annales des Sciences Naturelles, Botanique* **6**, 357-375.

Stearns SC, Hoekstra RF (2001) *Evolution: an introduction* Oxford University Press, New York.

Suc JP, Bertini A, Combourieu-Nebout N, *et al.* (1995) Structure of West Mediterranean vegetation and climate since 5.3 Ma. *Acta Zoologica Cracoviensia* **38**, 3-16.

Ukraintseva VV (1993) Pollen morphology of the family Cistaceae in relation to its taxonomy. *Grana* **Supplement 2**, 33-36.

Vargas P, Roselló JA, Oyama R, Güemes J (2004) Molecular evidence for naturalness of genera in the tribe Antirrhineae (Scrophulariaceae) and three independent evolutionary lineages from the New World and the Old. *Plant Systematic and Evolution* **249**, 151-172.

Wendel J, Doyle J (2005) Polyploidy and evolution in plants. In: *Plant diversity and evolution: genotypic and phenotypic variation in higher plants* (ed. Henry J), pp. 97-117. CABI publishing, Wallingford, UK.

Willkomm M (1856) *Cistinearum orbis veteris descriptio monographica. Icones et Descriptiones Plantarum.*

Zhang Q, Chiang TY, George M, Liu JQ, Abbott RJ (2005) Phylogeography of the Qinghai-Tibetan Plateau endemic *Juniperus przewalskii* (Cupressaceae) inferred from chloroplast DNA sequence variation. *Molecular Ecology* **14**, 3513-3524.

Apéndices

1

Estudio comparativo del éxito reproductor de *Cistus ladanifer* var. *ladanifer* y var. *maculatus*

Resumen

Las características florales (como forma, tamaño, color, olor) son interpretadas como caracteres adaptativos que evolucionan mediante fuerzas selectivas originadas por los polinizadores. *Cistus ladanifer* (Cistaceae) es un arbusto típicamente mediterráneo que presenta flores altamente poliníferas dependientes de los insectos para ser polinizadas. En la especie se distinguen dos morfos para el color del pétalo originados por la presencia (variedad *maculatus*) o ausencia (variedad *ladanifer*) de una mácula púrpura oscura en la base del pétalo. En este trabajo estimamos la posible variación en el éxito reproductor a nivel de flor (número de flores producidas) y a nivel de fruto (*fruit set* y *seed set*) en ambas variedades. La producción de flores, frutos y semillas fue muy elevada en todas las poblaciones lo que favorece la formación de un banco de semillas en el suelo característico de una especie "semillera obligada" como es la jara pringosa. Nuestros resultados parecen no mostrar discriminación entre morfos por parte de los polinizadores pues no se encontraron diferencias significativas entre variedades en ninguna de las variables estudiadas.

Palabras clave: *fruit set*, jara pringosa, *seed set*, polinización, selección natural

1. Introducción

Muchos de los rasgos morfológicos de las flores responden a la necesidad de atraer a los animales que las polinizan ya que el aumento de atractivo hacia los polinizadores conlleva un incremento del *fitness* de la planta. Los polinizadores responden a la variación en caracteres florales como el tamaño de la flor, olor, y color (Harding & Mankinen 1967; Kauffield & Sorenson 1971; Waser & Price 1981; Galen 1985; Stanton 1987; Stanton & Preston 1988; Herrera 1993; Conner & Rush 1996; Celedon-Neghme *et al.* 2007). En ciertas circunstancias (por ejemplo, competencia por polinizadores) pueden actuar fuerzas selectivas implicadas en el incremento de atractivo que aseguren más visitas por parte de los polinizadores. De este modo, las interacciones planta-polinizador pueden influir en los procesos microevolutivos (Darwin 1859; Grant 1949) ya que la preferencia de los polinizadores por un determinado morfo podría favorecer la selección de caracteres florales (Campbell 1989; Caruso 2000; Irwin & Strauss 2005) produciéndose cambios genéticos en las poblaciones polimórficas. La variación del color de los pétalos ha sido descrita en un gran número de plantas cuya polinización está mediada por animales (Kay 1978; Gómez 2000), comprobándose que individuos con colores más marcados reciben una mayor cantidad de visitas que los que presentan pétalos con colores pálidos o albinos (Waser & Price 1981).

A pesar de la importancia ecológica de las Cistáceas pocos estudios sobre su biología de la reproducción se han llevado a cabo (Herrera 1987; Brandt & Gottsberger 1988; Bosch 1992; Herrera 1992; Talavera *et al.* 1993). En concreto dichos trabajos se han basado principalmente en describir características florales (diámetro de las flores, número de estambres y carpelos por flor, cantidad de polen y primordios producidos, etc.), determinar el sistema de reproducción y estimar el éxito reproductor (cuantificar la producción de flores, la eficacia de la polinización y la transformación de primordios en semillas). Los pétalos de las flores de las Cistáceas presentan una coloración muy homogénea entre individuos pudiendo ser de color blanco, amarillo o rosa-púrpura. Independientemente del color del pétalo todas las especies presentan tonalidades amarillas en la uña del pétalo, pero solo algunas (p.ej., *Cistus ladanifer, Tuberaria guttata,*

Halimium halimifolium) presentan además una mácula de color oscuro en la base del pétalo que da lugar a la aparición de dos morfos bien diferenciados para el color del pétalo. No hemos encontrado ningún trabajo que evalúe si se manifiestan ventajas o desventajas entre individuos con flores maculadas y no maculadas. El principal objetivo de este trabajo es evaluar la importancia de la mácula púrpura del pétalo en la biología reproductiva de *C. ladanifer* y analizar si los dos morfos de color difieren en el éxito reproductor, analizando la producción de flores por individuos así como la fructificación y la transformación de primordios seminales en semillas.

2. Material y métodos

2.1. Características de la especie estudiada

Cistus ladanifer L., es un arbusto que habita en zonas con suelos pobres y ambientes soleados de un área comprendida entre el sur de Francia, Portugal y España hasta el norte de Marruecos y Argelia (Fig. 1A) (Demoly & Montserrat 1993). Sus flores solitarias, terminales y muy grandes (5-8 cm) (las más grandes en la flora mediterránea después de *Paeonia*) presentan tres sépalos y cinco pétalos de color blanco con una mancha amarilla en la base, y a veces otra purpúrea oscura superpuesta que ha dado lugar a la descripción de dos variedades: *C. ladanifer* var. *ladanifer* (Fig. 2A) y *C. ladanifer* var. *maculatus* Dunal (Fig. 2B) (Rouy & Foucaud 1895). Ambas variedades presentan los mismos caracteres vegetativos y pueden crecer entremezcladas en las mismas localidades y poblaciones.

Las flores desarrollan estambres numerosos (>100), desiguales en longitud y situados en torno a un estigma grande y sésil. Al igual que el resto de representantes de la familia, la jara pringosa presenta típicas "flores de polen" (Herrera 1987) que dependen de los insectos para ser polinizadas (principalmente abejas y escarabajos; Herrera 1988). La principal recompensa floral en Cistáceas es el polen, si bien es cierto que las especies del género *Cistus* presentan un disco nectarífero bajo el ovario (Janchen 1925; Herrera 1985; Bosch 1992) que da lugar a pequeñas cantidades de néctar, sustanciales en *C. ladanifer* (Talavera *et al.* 1993).

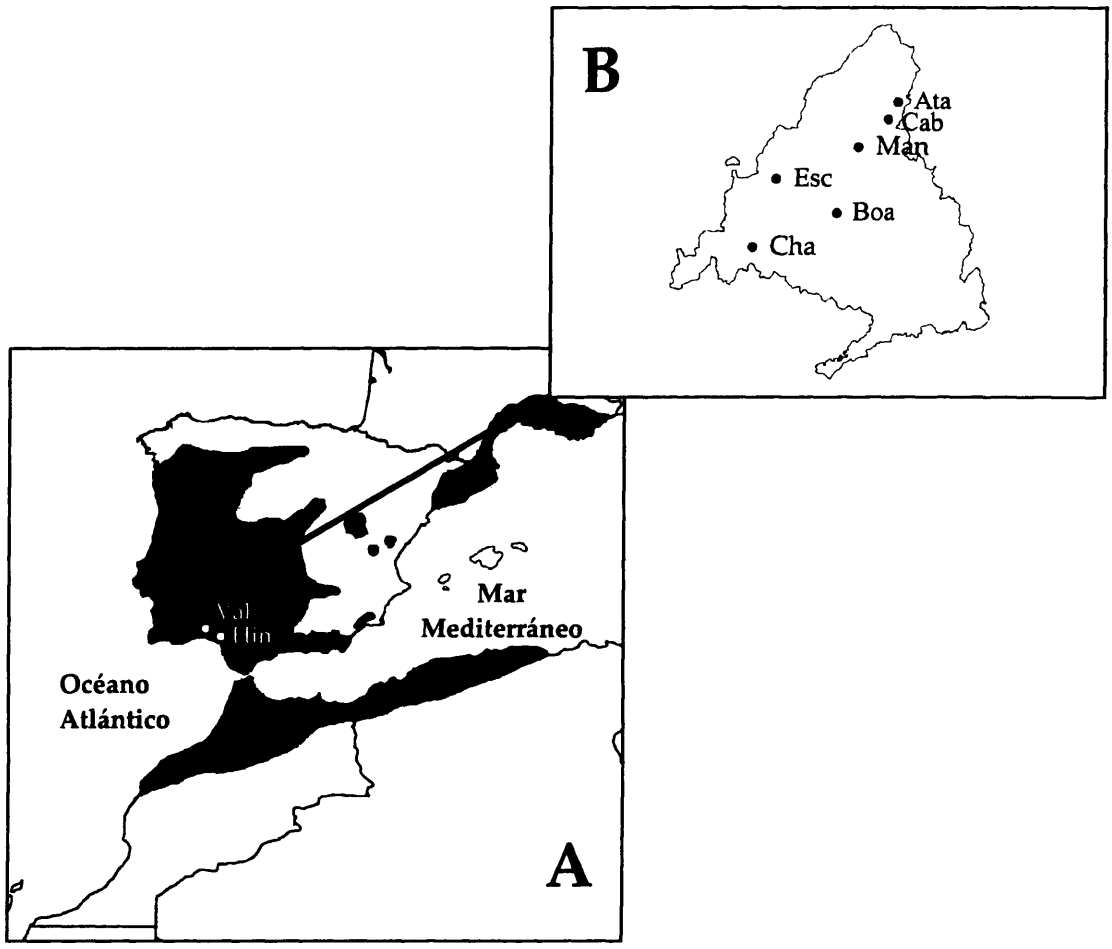

Figura 1. Área de distribución (A) y localización geográfica de las ocho poblaciones de jara pringosa estudiadas en la provincia de Huelva (A) y en la provincia de Madrid (B). Ver código de poblaciones en Tabla 1.

2.2. Experimentos de campo

El estudio se realizó entre los años 2003 y 2006 en ocho poblaciones de la Península Ibérica (Fig. 1A y 1B), donde la jara pringosa es la especie dominante. En la primavera del 2003 se realizaron cruzamientos recíprocos entre 10 individuos en seis poblaciones de la Sierra de Guadarrama (Madrid) y dos de Andalucía (Huelva) (Tabla 1). Para ello se seleccionaron al azar poblaciones monotípicas de *C. ladanifer* (en las poblaciones donde individuos de las dos variedades coexistían se seleccionaron individuos de la variedad predominante). Para evitar problemas de consanguinidad, los cruzamientos fueron realizados entre individuos de poblaciones separadas entre sí al menos 20 Km en

línea recta. Siguiendo el método de polinización descrito por Gard (1910) y Talavera *et al.* (1993), en cada individuo fueron polinizadas seis-ocho flores. Cinco-seis de ellas se polinizaron con polen de individuos de otra población y, por tanto, de otra variedad (Tabla 1) y dos fueron autopolinizadas. El test de autoincompatilibidad se realizó sólo en las poblaciones de Madrid. Además, en cada individuo, se dejaron dos flores sin ningún tratamiento, considerándolas como polinización libre.

Tabla 1. Código de población, variedad y localización geográfica de las poblaciones de jara pringosa estudiadas

CÓDIGO	VARIEDAD	LOCALIDAD	LATITUD	LONGITUD
Ata	*maculatus*	El Atazar, Madrid	40° 55′ N	3° 28′ W
Cab	*ladanifer*	La Cabrera, Madrid	40° 52′ N	3° 37′ W
Man	*maculatus*	Manzanares el Real, Madrid	40° 43′ N	3° 52′ W
Esc	*ladanifer*	El Escorial, Madrid	40° 34′ N	4° 07′ W
Cha	*maculatus*	Chapinería, Madrid	40° 22′ N	4° 12′ W
Boa	*ladanifer*	Boadilla del Monte, Madrid	40° 23′ N	3° 52′ W
Val	*maculatus*	Valverde del Camino, Huelva	37° 34′ N	6° 45′ W
Hin	*ladanifer*	Hinojos, Huelva	37° 17′ N	6° 22′ W

Al final del periodo de floración en cada individuo se realizaron estimas de las siguientes variables: 1) número total de flores producidas; 2) número total de frutos maduros y 3) número de primordios seminales no desarrollados y semillas contenidos en dos valvas opuestas de 3-4 frutos seleccionados al azar. Posteriormente, se calculó la producción de frutos o *fruit set* (número de frutos/número de flores) de cada individuo y la proporción de primordios transformados en semillas o *seed set* (número de semillas/número de primordios producidos) de cada fruto, que fue estimada multiplicando el número total de valvas de cada fruto (que es muy variable en *C. ladanifer*; véase capítulo 7) por el valor obtenido de las dos valvas analizadas (Talavera *et al.* 1993). Estas variables (*fruit set* y *seed set*) se calcularon para todas las poblaciones excepto para la de Chapinería (Madrid).

En al año 2006, para estimar si la presencia de mácula en el pétalo afecta al *seed set* de los individuos de poblaciones mixtas se recolectaron al azar cinco frutos de 20 individuos (10 individuos de cada variedad) en cada una de las poblaciones de El Escorial y La Cabrera.

Figura 2. Aspecto de las dos variedades de *Cistus ladanifer*: var. *ladanifer* (A) y var. *maculatus* (B). Fotografías realizadas por B. Guzmán.

2.3. Análisis estadísticos

La diferencia en la eficacia de la polinización libre y manual fue analizada mediante una prueba de la t de Student. Las posibles diferencias en el número de flores por individuo así como en el *fruit set* y *seed set* de siete poblaciones de las dos variedades de *C. ladanifer* fueron analizadas a través de un ANOVA encajado donde el factor población fue considerado como aleatorio y encajado en el factor variedad. Las diferencias de *seed set* entre individuos de las dos variedades en poblaciones mixtas fueron analizadas

mediante un ANOVA factorial en el que el factor población fue considerado como aleatorio.

La homogeneidad de varianzas se comprobó mediante el test de Levene (Day & Quinn 1989; Statsoft 1999). Para saber si la distribución de los datos se ajustaba a la función normal se utilizó el test de bondad de ajuste de Kolmogorov-Smirnov con la corrección de Lilliefors (Statsoft 1999). También fueron comprobadas la normalidad e independencia de los residuos. Los datos relativos al *fruit set* y *seed set* fueron transformados mediante arcoseno. Todos los análisis estadísticos fueron llevados a cabo con el programa informático STATISTICA 6.0 (Statsoft, Incorporated, Tulsa, Oklahoma, USA), excepto la estimación de remuestreo para las representaciones gráficas que fue realizada con la macro del programa Excel (DataPilot 1.03) desarrollada por TwoPilot Inc.

3. Resultados

En todos los cruzamientos realizados se obtuvieron frutos (Tabla 2) aunque la eficacia de la polinización manual (59,1 ± 37,7%) resultó ser significativamente más baja que la de la polinización libre (80,7 ± 16,8) (t-test: gl = 108, t = -3,7, p = 0,0003). Dicho descenso en el *fruit set* ha sido documentado para otras especies, y puede estar causado por la interferencia entre la gran densidad de tubos polínicos o por que la polinización manual se realizara cuando el estigma no estaba en su óptimo de receptividad (Young & Young 1992). Por otra parte, ningún fruto se obtuvo de las autopolinizaciones realizadas (pero véase apéndice 2).

Tabla 2. Resultado de las polinizaciones libres estimadas en diez individuos (media ± desviación estándar), cruzadas y autopolinizaciones de seis poblaciones de la Comunidad de Madrid y dos de Andalucía. Poblaciones de *Cistus ladanifer* variedad *maculatus* indicadas en negrita

	Polinizaciones libres[1]		**Polinizaciones cruzadas**		**Autopolinizaciones[1]**	
	N° flores	N° frutos	N° flores	N° frutos	N° flores	N° frutos
Atazar x **Cabrera**	184,3 ± 158,9	132,4 ± 134,3	5,1 ± 0,32	4,1 ± 1,2	1,9 ± 0,3	0
Cabrera x Atazar	169,3 ± 141,4	1372 ± 104,0	4,9 ± 0,32	4, 1 ± 1,7	2,0 ± 0,0	0
Manzanares x **Escorial**	220,0 ± 125,6	163,6 ± 409,0	5,0 ± 0,0	2,6 ± 1,6	2,0 ± 0,0	0
Escorial x Manzanares	208,5 ± 148,0	198,0 ± 143,7	6,0 ± 0,0	4,0 ± 2,3	2,0 ± 0,0	0
Boadilla x **Chapinería**	178,1 ± 96,1	163,9 ± 94,3	5,2 ± 0,6	0,6 ± 1,3	2,0 ± 0,0	0
Chapinería x Boadilla	-	-	5,8 ± 0,4	3,6 ± 1,7	2,0 ± 0,0	0
Hinojos x **Valverde**	229,4 ± 119,2	185,0 ± 118,4	6 ± 0,0	-	-	-
Valverde x Hinojos	564,0 ± 42,21	42,0 ± 36,9	6 ± 0,0	-	-	-

[1] Datos correspondientes a la primera población de cada cruzamiento

Los ocho cruzamientos intermorfos resultaron ser viables obteniéndose de todos ellos una generación F1 que al cabo de 4 años sigue sin florecer (70 plantas), de manera que la línea de investigación sobre el control genético de la mácula sigue abierta no pudiéndose incluir ningún resultado ni conclusión al respecto en la presente memoria doctoral.

En la primavera de 2003, las plantas de la variedad *maculatus* produjeron entre 31 y 528 flores, mientras que las de la variedad *ladanifer* produjeron entre 11 y 531 flores (Fig. 3A). El ANOVA realizado reveló que las diferencias halladas entre poblaciones y variedades no son estadísticamente significativas (Tabla 3A), de manera que la producción de flores parece estar correlacionada con el tamaño/edad y posición de la planta (aislada o en grupos) (Talavera *et al.* 1993) más que con la variedad a la que pertenece. Todas las poblaciones presentaron porcentajes de fructificación relativamente elevados, oscilando entre el 18,8% y el 97,3% en los individuos de poblaciones sin mácula y entre el 45,5% y el 97,0% en los individuos de las maculadas (Fig. 3B). Además no se encontraron diferencias estadísticamente significativas entre ambas variedades (Tabla 4B). El porcentaje medio de transformación de primordios seminales en semillas fue de 90 ± 8,8% y 94 ± 3,5% para las poblaciones con pétalos no maculados y maculados respectivamente (Fig. 3C). Al igual que ocurre con la

fructificación no existieron diferencias estadísticamente significativas entre ambas variedades (Tabla 3C).

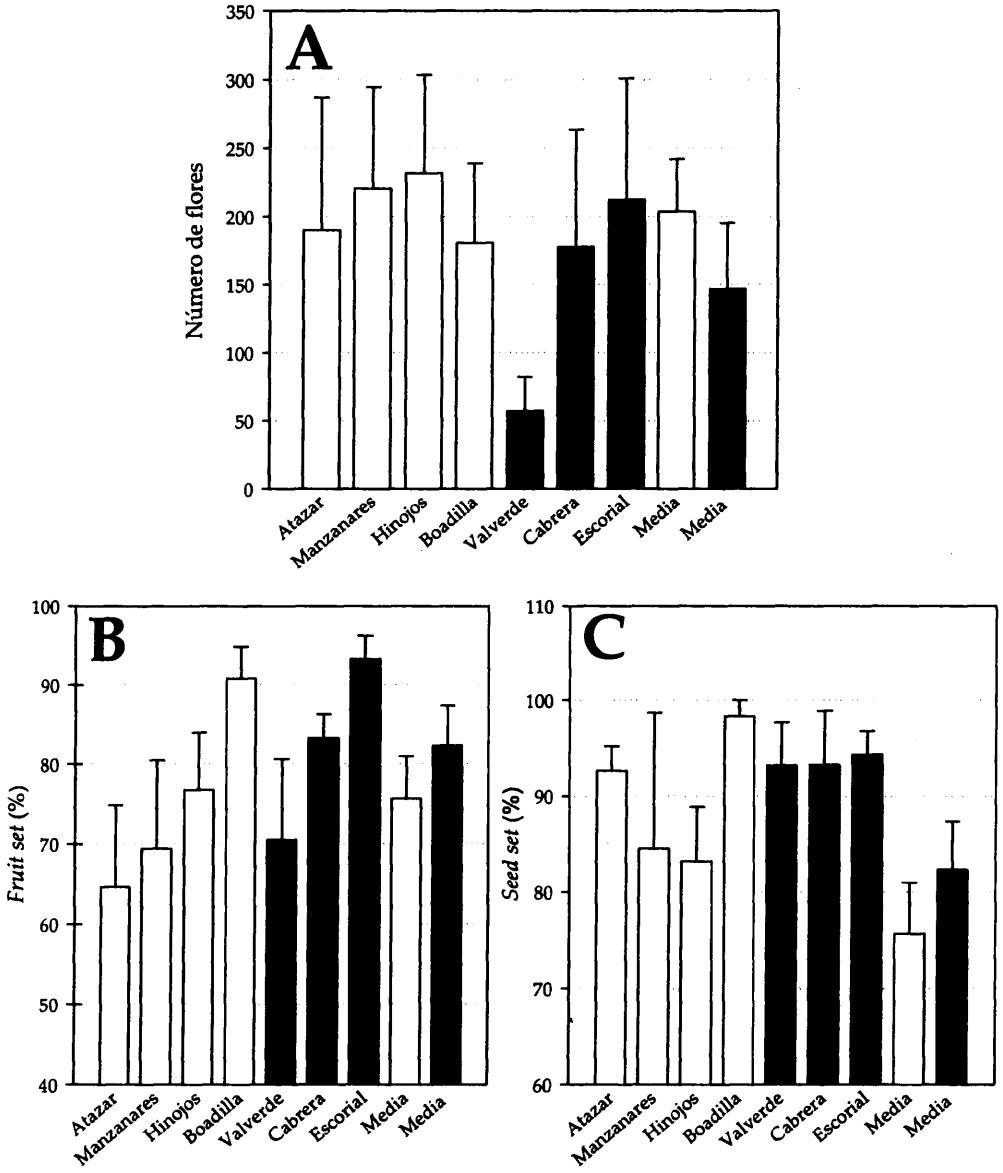

Figura 3. Valor medio (± intervalos de confianza) del número de flores (A), número de flores transformadas en frutos o *fruit set* (B) y número de primordios seminales transformados en semillas o *seed set* (C) encontrado en siete poblaciones de jara pringosa localizadas en la provincia de Madrid y Huelva ($P < 0,05$, estimado mediante remuestreo, 100.000 réplicas). Ver en Tabla 3 resultados estadísticos. Las barras blancas corresponden a poblaciones de *Cistus ladanifer* var. *maculatus* y las barras negras a poblaciones de *Cistus ladanifer* var. *ladanifer*.

Tabla 3. Análisis de varianza evaluando el efecto de la variedad y de la población (encajado a la variedad) en el número de flores del individuo, *fruit set* y *seed set* en siete poblaciones de la Sierra de Guadarrama (Madrid) y Huelva. *gl* = grados de libertad; *SC* = Suma de Cuadrados

	gl	*SC*	*F*	*p*
A. FLORES PRODUCIDAS				
Variedad	1	58.100	2,01	0,21
Población (Variedad)	5	144.281	1,87	0,11
Error	63	972.324		
B. *FRUIT SET*				
Variedad	1	0,21	0,53	0,50
Población (Variedad)	5	2,01	10,05	<0,0001
Error	63	2,52		
C. *SEED SET*				
Variedad	1	0,02	0,21	0,66
Población (Variedad)	5	0,40	4,19	0,006
Error	27	0,52		

En el 2006, el *seed set* encontrado en las dos poblaciones mixtas estudiadas fue elevado en los individuos de las dos variedades (La Cabrera: 85,5 ± 14,17% en individuos con pétalos maculados y 87,5 ± 9,80% en individuos con pétalos no maculados; El Escorial: 85,4 ± 13,67% en individuos con pétalos maculados y 82,5 ± 18,54% en individuos con pétalos no maculados) y no fue significativamente diferente en ninguno de los dos niveles de variación estudiados: población y variedad (Tabla 4).

Tabla 4. Resultados del ANOVA factorial realizado para investigar el efecto de la mácula del pétalo en el *seed set* de dos poblaciones de *C. ladanifer* de la Sierra de Guadarrama (Esc, Cab). *gl* = grados de libertad; *SC* = Suma de Cuadrados

	gl	*SC*	*F*	*p*
Población	1	202521	5.02	0.27
Variedad	1	155658	3.86	0.30
Población*Variedad	1	40344	0.46	0.49
Error	191	16554454		

4. Discusión

Hasta la fecha, los trabajos de *Cistus ladanifer* que conocemos estudiaron el éxito reproductor en una única población (Herrera 1992; Talavera *et al.* 1993; Blasco & Mateu 1995; Talavera *et al.* 2001). En nuestro estudio comparado (7 poblaciones), *Cistus ladanifer* presentó diferencias significativas entre poblaciones, tanto en el porcentaje de fructificación ($F_{5,\ 2,01}$ = 10,05; $P < 0.0001$) como en el de transformación de primordios seminales en semillas ($F_{5,\ 0,40}$ = 4,09; P = 0,006), por lo que se hace evidente la necesidad del estudio de varias poblaciones para poder obtener conclusiones generales respecto al éxito reproductor de esta especie.

La transformación de primordios seminales en semillas fue superior al 80% en las flores no manipuladas de todas las poblaciones. Este porcentaje es muy elevado, al igual que ocurre en otras especies del género (*C. libanotis*, Talavera *et al.* 1993; Talavera *et al.* 2001) que, como la jara pringosa son "semilleras obligadas" (*sensu* Keeley 1991). Mediante un elevado *fruit set* y *seed set* la planta da lugar a la formación en el suelo de un banco de semillas (Thanos *et al.* 1992) que asegura su dispersión en el tiempo como adaptación a las impredecibles perturbaciones ambientales ocurridas en los ecosistemas Mediterráneos (p.ej. fuego).

El éxito reproductor de las angiospermas a menudo depende de la polinización por insectos. Una cuestión inmediata a investigar es plantear experimentos específicos para analizar la posible discriminación de los polinizadores ante los dos fenotipos florales (var. *maculatus*, var. *ladanifer*), puesto que la presencia o ausencia de la mácula podría influir en el atractivo de la flor frente al insecto. La falta de diferencias significativas en el éxito reproductor (Tabla 3) de ambos morfos indicaría que los resultados finales en la producción de frutos y semillas son similares independientemente del atractivo de la flor ante los polinizadores. Los insectos perciben los colores de la banda espectral del ultravioleta próximo (el azul, el verde y el amarillo) pero solo algunos son sensibles al rojo (Menzel *et al.* 1986; Goldsmith 1990). En el esfuerzo por conocer las causas últimas de la formación de la mácula, la Dra. Carmen García-Cordovés analizó los compuestos químicos principales responsables del color. En concreto ella detectó la presencia de dos

pigmentos: el delfinidín-3-glucósido en un 15,9% y el cianidín-3-glucósido en un 84,1% (Fig. 4).

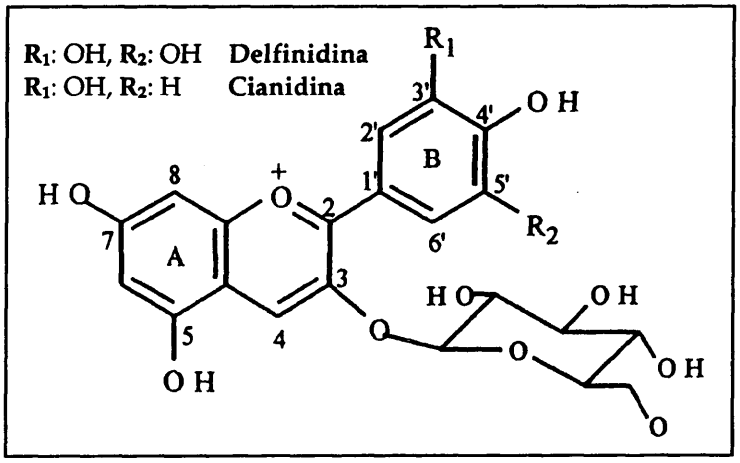

Figura 4. Estructura de los pigmentos antociánicos (delfinidín-3-glucósido y cianidín-3-glucósido) encontrados en extractos de mácula púrpura de *C. ladanifer* variedad *maculatus* mediante un estudio de espectrofotometría de masas.

Por otro lado, Arias *et al.* (1995) demostraron que las flores de ambas variedades de *Cistus ladanifer* eran vistas del mismo modo por los insectos, siendo la mácula púrpura de la variedad *maculatus* no detectada por ellos. Este hecho parece apoyar nuestro resultado de éxito reproductor similar entre las dos variedades aunque un estudio más exhaustivo sobre polinizadores debería realizarse.

En resumen, este estudio no ha podido determinar la función de la mácula y no aporta pruebas que señalen el valor adaptativo de la misma. La presencia de mácula muestra cierto patrón geográfico en el sur de la península Ibérica (var. *ladanifer* en suelos arenosos de los valles, var. *maculatus* en montaña), pero no en la Sierra de Guadarrama y resto de la Península donde las poblaciones suelen ser mixtas (Guzmán et al., datos sin publicar). Los patrones filogeográficos en *C. ladanifer* (capítulos 6 y 7) tampoco nos han permitido relacionar los linajes de las poblaciones con y sin mácula en un marco geográfico. No obstante, estudios con marcadores moleculares hipervariables (*fingerprinting*) y análisis de genética de las rutas metabólicas que conducen a la producción de mácula son necesarios para estimar el valor adaptativo de la misma

frente a la hipótesis de deriva génica. Asimismo, el hecho de que este carácter aparezca en distintos géneros de Cistáceas europeas permite plantear experimentos comparativos entre *C. ladanifer* y las especies maculadas de *Halimium* y *Tuberaria*.

Agradecimientos

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Referencias bibliográficas

Arias J, Carrera MT, Fernández L, Gutiérrez F, Moreno M (1995) Imagen de las flores de algunas Cistaceae ibéricas en el espectro de visión de los insectos. *Historia Natural'93*, 23-28.

Blasco S, Mateu I (1995) Flowering and fruiting phenology and breeding system of *Cistus albidus* L. *Acta Botanica Gallica* **142**, 245-251.

Bosch J (1992) Floral biology and pollinators of three co-occurring *Cistus* species (Cistaceae). *Botanical Journal of the Linnean Society* **109**, 39-55.

Brandt U, Gottsberger G (1988) Flower phenology. Pollinating insects and breeding systems in *Cistus, Halimium* and *Tuberaria* species in Portugal. *Lagascalia* **15**, 625-634.

Campbell DR (1989) Measurements of selection in a hermaphroditic plant: variation in male and female pollination success. *Evolution* **43**, 318-334.

Caruso CM (2000) Competition for pollination influences selection on floral traits of *Ipomopsis aggregata*. *Evolution* **54**, 1546-1557.

Celedon-Neghme C, Gonzáles WL, Gianoli E (2007) Cost and benefits of attractive floral traits in the annual species *Madia sativa* (Asteraceae). *Evolutionary Ecology* **21**, 247-257.

Conner JK, Rush S (1996) Effects of flower size and number on pollinator visitation to wild radish *Raphanus raphanistrum*. *Oecologia* **105**, 509-516.

Darwin C (1859) *The Origin of Species by Means of Natural Selection*, London.

Day RW, Quinn GP (1989) Comparisons of treatments after an analysis of variance in ecology. *Ecological Monographs* **59**, 433-463.

Demoly JP, Montserrat P (1993) *Cistus*. In: *Flora iberica* (eds. Castroviejo S, *et al.*), pp. 319-337. Consejo de Investigaciones Científicas, Madrid.

Galen C (1985) Regulation of seed-set in *Polemonium viscosum*: floral scents, pollination, and resources. *Ecology* **66**, 792-797.

Gard M (1910) Recherches sur les hybrides artificiels des Cistes, obtenus par M. Ed. Bornet. Premier mémoire: Notes inédites et résultats expérimentaux. *Annales des Sciences Naturelles, Botanique* **9**, 71-115.

Goldsmith TH (1990) Optimization, constraint, and history in the evolution of eyes. *Quarterly Review of Biology* **65**, 281-322.

Gómez JM (2000) Phenotypic selection and response to selection in *Lobularia maritima*: importance of direct and correlational components of natural selection. *Journal of Evolutionary Biology* **13**, 689-699.

Grant V (1949) Pollination systems as isolating mechanisms in angiosperms. *Evolution & Development* **3**, 82-97.

Harding J, Mankinen CB (1967) Genetics of *Lupinus*. I. Variations in flower color from natural populations of *Lupinus nanus*. *Canadian Journal of Botany* **45**, 1831-1836.

Herrera CM (1993) Selection on floral morphology and environmental determinants of fecundity in a hawkmoth-pollinated violet. *Ecological Monographs* **63**, 251-275.

Herrera J (1985) Nectar secretion patterns in Southern Spanish Mediterranean scrubland. *Israel Journal of Botany* **34**, 47-58

Herrera J (1987) Flower and fruit biology in southern spanish Mediterranean shrublands. *Annals of the Missouri Botanical Garden* **74**, 69-78.

Herrera J (1988) Pollination relationships in sourthern Spanish Mediterranean shrublands. *Journal of Ecology* **76**, 274-287.

Herrera J (1992) Flower variation and breeding systems in the Cistaceae. *Plant Systematics and Evolution* **179**, 245-255.

Irwin RE, Strauss SY (2005) Flower color microevolution in wild radish: Evolutionary response to pollinator-mediated selection. *The American naturalist* **165**, 225-237.

Janchen E (1925) Cistaceae. In: *Die natürlichen Pflanzenfamilien* (eds. Engler A, Prantl K), pp. 289-313. W. Engelmann, Leipzig.

Kauffield NM, Sorenson EL (1971) Interrelation of honeybee preference for alfalfa clones differing in flower color, aroma, nectar volume, and sugar concentration. *Kansas Agricultural Experiment Station Research Publication* **163**, 1-14.

Kay QQN (1978) The role of preferential and assortative pollination in the maintenance of flower color polymorphisms. In: *The pollination of flowers by insects* (ed. Richards AJ), pp. 175-190. Academic Press, London.

Keeley JE (1991) Seed germination and life history syndromes in the California Chaparral. *Botanical Review* **57**, 81-116.

Menzel R, Ventura DF, Hertel H, de Souza J, Greggers U (1986) Spectral sensitivity of photoreceptors in insect compound eyes: Comparison of species and methods. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral* **158**, 165-177.

Rouy G, Foucaud J (1895) Cistinées. In: *Flore de France* (ed. Rouy G), pp. 254-316.

Stanton ML (1987) The reproductive biology of petal color variants in wild populations of *Raphanus sativus*: I. Pollinator response to color morphs. *American Journal of Botany* **74**, 178-187.

Stanton ML, Preston RE (1988) Ecological consequences and phenotypic correlates of petal size variation in wild radish, *Raphanus sativus* (Brassicaceae). *American Journal of Botany* **75**, 528-539.

Statsoft (1999) STATISTICA for Windows (Computer Program Manual). In: *Statsoft, Inc.*, Statsoft, Inc., Tucsa.

Talavera S, Bastida F, Ortiz PL, Arista M (2001) Pollinator attendance and reproductive success in *Cistus libanotis* L. (Cistaceae). *International Journal of Plant Sciences* **162**, 343-352.

Talavera S, Gibbs PE, Herrera J (1993) Reproductive biology of *Cistus ladanifer* (Cistaceae). *Plant Systematics and Evolution* **186**, 123-134.

Thanos CA, Georghiou K, Kadis C, Pantazi C (1992) Cistaceae: A plant family with hard seeds. *Israel Journal of Botany* **41**, 251-263.

Waser NM, Price MV (1981) Pollinator choice and stabilizing selection for flower color in *Delphinium nelsonii*. *Evolution* **35**, 376-390.

Young HJ, Young TP (1992) Alternative outcomes of natural and experimental high pollen loads. *Ecology* **73**, 639-647.

2

Estudio temporal de autoincompatibilidad en *Cistus ladanifer*

La mayoría de las plantas son hermafroditas por lo que potencialmente podrían autopolinizarse (Yampolsky & Yampolsky 1922). A pesar de las ventajas que ofrece la autopolinización (reproducción asegurada aún cuando los polinizadores son escasos, colonización de nuevas áreas a partir de un único individuo, etc.) aproximadamente la mitad de las familias de las angiospermas incluyen especies con algún tipo de autoincompatibilidad (heteromórfica, homomórfica, gametofítica, esporofítica; De Nettancourt 1977). En algunas especies el estado de autoincompatibilidad puede variar en función de: (1) condiciones ambientales, (2) condiciones intrínsecas con la edad o (3) mutaciones que afectan a los genes que determinan este carácter (Levin 1996; Stephenson *et al.* 2000; Good-Avila & Stephenson 2002; Tsukamoto *et al.* 2003a; Tsukamoto *et al.* 2003b). Al final del periodo de floración se dan ciertas condiciones, como la edad floral y variaciones de temperatura, que unidas a una posible disminución en la producción de frutos (*fruit set*), pueden producir una flexibilidad en el sistema de reproducción solo posible cuando la mayoría de las oportunidades de cruzamiento han pasado (Lloyd & Schoen 1992). Esta condición, denominada "autocompatibilidad retrasada" (Lloyd & Schoen 1992), ha sido observada en *Campanula rapunculoides* (Vogler *et al.* 1998) vinculada a la edad floral y a la cantidad de frutos desarrollados por la planta.

La gran mayoría de los representantes de la familia Cistaceae presentan una autoincompatibilidad homomórfica gametofítica (Janchen 1925; Brandt & Gottsberger 1988; Herrera 1992; Talavera *et al.* 1993). Sin embargo Morse (1979) describió la existencia de autogamia facultativa en el género *Hudsonia* y Bosch (1992) propuso que *Cistus albidus*, al igual que otros miembros del género (Herrera 1987, 1992), podían transformarse en una especie autocompatible bajo determinadas circunstancias, pero sin especificar cuáles.

En la primavera del año 2004, en cuatro poblaciones de la Comunidad de Madrid (El Atazar, La Cabrera, El Escorial y Manzanares El Real) utilizadas para el estudio comparado entre variedades de *Cistus ladanifer* (ver Apéndice 1 (año 2003)) se realizó el test de autoincompatibilidad en 1-2 flores de 9-10 individuos (mayormente los

individuos coinciden con los del año 2003). El porcentaje de fructificación obtenido (6,25-37,5%; Tabla 1) resultó no ser el esperado (Talavera *et al.* 1993 y apéndice 1). A diferencia del año 2003 (cuando un 0% de fructificación fue obtenido en las autopolinizaciones), en el año 2004 el test se realizó al final del periodo de floración de la especie en la Comunidad de Madrid (finales del mes de mayo), por lo que pensamos investigar si éste era el motivo de dicho aumento en la fructificación. Por este motivo en la primavera del año 2006 se realizó un estudio de autoincompatibilidad para testar si la jara pringosa mantiene esta potencialidad a lo largo de todo el periodo de su fenología floral. En las poblaciones de El Escorial y La Cabrera (Sierra de Guadarrama, Madrid) se seleccionaron aleatoriamente 30 individuos en los que se polinizaron, a lo largo del mes de mayo, un número comprendido entre 4-37 flores. Tanto en el año 2004 como en el 2006 los test de autoincompatibilidad se realizaron con polen de la propia flor y con polen de otras flores del mismo individuo siguiendo metodologías descritas por Gard (1910) y Talavera *et al.* (1993).

Tabla 1. Resultado de los test de autoincompatibilidad realizados en la primavera de los años 2004 y 2006 en cuatro y dos poblaciones, respectivamente, de la Comunidad de Madrid

	2004			2006		
	N° flores autopolinizadas	**N° frutos producidos**	**Fructificación (%)**	**N° flores autopolinizadas**	**N° frutos producidos**	**Fructificación (%)**
La Cabrera	16	6	37.5	742	3	0.4
El Escorial	19	3	15.8	878	1	0.1
El Atazar	16	1	6.3	-	-	-
Manzanares	15	1	6.6	-	-	-

De las 742 y 878 flores autopolinizadas, respectivamente, en las poblaciones de La Cabrera y El Escorial únicamente se obtuvieron cuatro frutos (Tabla 1). El bajo número de frutos obtenido y la morfología de éstos (frutos muy pequeños con un bajo número de semillas) nos permiten decir que los individuos de ambas poblaciones fueron predominantemente autoincompatibles independientemente del "momento" en la fenología floral en el que se encuentran. Esto nos hace pensar que la fructificación

obtenida en el año 2004 se debió a un problema metodológico al no emplear en las autopolinizaciones polen de la propia planta. En ocasiones lo que *a priori* es considerado un único arbusto puede estar formado por tallos que aún partiendo a la misma base pertenecen a individuos distintos. Por tanto, estaríamos realizando cruces xenógamos en vez de autopolinizaciones.

En resumen, la potencialidad de una autocompatibilidad retrasada en *Cistus ladanifer* es considerada realmente baja sobre la base de nuestros resultados de nula o baja producción de frutos y semillas. Aunque en un trabajo previo no se aplica una escala temporal y espacial (Talavera *et al.* 1993), nuestros resultados apoyan los de dichos autores en la descripción de la especie como predominante autoincompatible.

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Referencias bibliográficas

Bornet ED, Gard M (1910) Recherches sur les hybrides artificiels des Cistes. Premier mémoire: Notes inédites et résultats expérimentaux. *Annales des Sciences Naturelles, Botanique* **9**, 71-115.

Bosch J (1992) Floral biology and pollinators of three co-occurring *Cistus* species (Cistaceae). *Botanical Journal of the Linnean Society* **109**, 39-55.

Brandt U, Gottsberger G (1988) Flower phenology. Pollinating insects and breeding systems in *Cistus, Halimium* and *Tuberaria* species in Portugal. *Lagascalia* **15**, 625-634.

De Nettancourt D (1977) *Incompatibility in Angiosperms* Springer, New York.

Good-Avila SV, Stephenson AG (2002) The inheritance of modifiers conferring self-fertility in the partially self-incompatible perennial, *Campanula rapunculoides* L. (Campanulaceae). *Evolution* **56**, 263-272.

Herrera J (1987) Flower and fruit biology in southern spanish Mediterranean shrublands. *Annals of the Missouri Botanical Garden* **74**, 69-78.

Herrera J (1992) Flower variation and breeding systems in the Cistaceae. *Plant Systematics and Evolution* **179**, 245-255.

Janchen E (1925) Cistaceae. In: *Die natürlichen Pflanzenfamilien* (eds. Engler A, Prantl K), pp. 289-313. W. Engelmann, Leipzig.

Levin DA (1996) The evolutionary significance of pseudo-self-fertility. *The American Naturalist* **148**, 321-332.

Lloyd DG, Schoen DJ (1992) Self- and cross-fertilization in plants. I. Functional dimensions. *International Journal of Plant Sciences* **153**, 358-369.

Morse LE (1979) *Systematics and ecological biogeography of the genus Hudsonia (Cistaceae), the sand heather*, Harvard University.

Stephenson AG, Good SV, Vogler DW (2000) Interrelationships among inbreeding depression, plasticity in the self-incompatibility system, and the breeding system of *Campanula rapunculoides* L. (Campanulaceae). *Annals of Botany* **85**, 211-220.

Talavera S, Gibbs PE, Herrera J (1993) Reproductive biology of *Cistus ladanifer* (Cistaceae). *Plant Systematics and Evolution* **186**, 123-134.

Tsukamoto T, Ando T, Kokubun H, et al. (2003a) Breakdown of self-incompatibility in a natural population of *Petunia axillaris* caused by modifier locus that suppresses the expression of an S-RNase gene. *Sexual Plant Reproduction* **15**, 255-263.

Tsukamoto T, Ando T, Takahashi M, et al. (2003b) Breakdown of self-incompatibility in a natural population of *Petunia axillaris* caused by a loss of pollen function. *Plant Physiology* **131**, 1903-1912.

Vogler DW, Das C, Stephenson AG (1998) Phenotypic plasticity in the expression of self-incompatibility in *Campanula rapunculoides*. *Heredity* **81**, 546-555.

Yampolsky C, Yampolsky H (1922) Distribution of sex forms in the phanerogamic flora. *Bibliotheca Genetica* **3**, 1-62.

3

Estudio de germinación de semillas en *Cistus ladanifer*

Para el estudio del ajuste de las poblaciones a unas condiciones determinadas (*fitness*) se estudian numerosas estimas. En realidad, las distintas estimas de *fitness* miden los ajustes de las poblaciones en ciertas fases y ante ciertas circunstancias. Rara vez se obtienen datos del ciclo completo de una generación y mucho menos de varias generaciones consecutivas. No obstante, hay ciertas fases críticas que repercuten con mayor impacto en el *fitness* final. Una vez analizados el *fruit set* y *seed set* en *C. ladanifer* bajo diferentes circunstancias (Apéndice 1), el presente apéndice aporta resultados correspondientes al siguiente paso del ciclo biológico: viabilidad de las semillas. En concreto, se ha realizado un estudio comparativo entre la viabilidad de las semillas en la escala de individuo, poblaciones y táxones (variedades) de *C. ladanifer*.

Para estudiar la variación en el porcentaje de germinación de semillas procedentes de siete poblaciones diferentes (cinco de la Comunidad de Madrid y dos de Andalucía), cuatro frutos (dos resultantes de una polinización libre y otros dos resultantes de polinizaciones manuales) de diez individuos fueron seleccionados al azar. Cada fruto produjo un número elevado de semillas (833,12-1018,43, véase capítulo 7). Tras ser pesadas, las semillas fueron pretratadas con calor seco para su germinación (10 minutos a 100 °C) (Corral *et al.* 1990; Keeley 1991; González-Rabanal & Casal 1995; Pérez-García 1995, 1997). De cada fruto se realizaron tres réplicas de 50 semillas que se colocaron en placas de Petri con discos de papel de filtro saturados con agua destilada. El experimento se llevó a cabo en una cámara de germinación modelo IBERCEX, con un fotoperiodo de 16 horas de luz (35 µmol m^-2 s^-1 PAR) a 23 °C y 8 horas de oscuridad a 13 °C. Las semillas germinadas (considerada la germinación cuando la radícula emerge de la testa de la semilla) fueron contadas y eliminadas de la placa diariamente.

Estudios empíricos han mostrado que el tamaño de las semillas puede afectar a la supervivencia, crecimiento y establecimiento de las mismas (Howe & Richter 1982; Winn 1988; Houssard & Escarré 1991; Eriksson 1999), resultando este factor un indicador de calidad de las semillas (McGinley *et al.* 1987; aunque ver Lehtilä & Syrjänen 1995). Para detectar el efecto de este factor y las posibles diferencias en la tasa de germinación entre las distintas poblaciones se realizó un análisis de covarianza

(ANCOVA). El peso de las semillas se empleó como covariable y los factores población e individuo fueron considerados aleatorios. Además, el factor población fue encajado en la variedad y el factor individuo en la variedad y la población. La existencia de diferencias en la tasa de germinación debidas al origen del fruto (polinización libre o manual) un ANCOVA con la variable tratamiento fue abordado.

La homogeneidad de varianzas se comprobó mediante el test de Levene (Day & Quinn 1989; Statsoft 1999). Para saber si la distribución de los datos se ajustaba a la función normal se utilizó el test de bondad de ajuste de Kolmogorov-Smirnov con la corrección de Lilliefors (Statsoft 1999). El peso de las semillas no pudo ser trasformado a una distribución normal, pero los F-test son robustos a este tipo de desviaciones cuando N es suficientemente grande (Lindman 1974; Zar 1999). Todos los análisis estadísticos fueron llevados a cabo con el programa informático STATISTICA 6.0 (Statsoft, Incorporated, Tulsa, Oklahoma, USA), excepto la estimación de remuestreo para las representaciones gráficas que fue realizada con la macro del programa Excel (DataPilot 1.03) desarrollada por TwoPilot Inc.

Diferencias en la tasa de germinación de semillas procedentes de frutos obtenidos por polinización libre o manual resultaron no ser estadísticamente significativas ($F_{1, 0.01}$ = 0,11, P = 0,73). La proporción de semillas germinadas fue moderadamente elevada (57,20-78,82%) en todas las poblaciones (Fig. 1). La comparación del porcentaje de germinación entre individuos, poblaciones y variedades reveló la existencia de diferencias significativas únicamente entre individuos (Tabla 1). Por otro lado, aunque el peso mostró tener un efecto positivo en la germinación de las semillas (cuanto más pesadas son, más fácilmente germinarán las semillas), el efecto resultó ser ligeramente significativo (Tabla 1). Además, la interacción de la variedad y el peso de las semillas no resultó ser significativa lo que implica que la relación entre la tasa de germinación y el peso de las semillas es constante, es decir no varía entre plantas con o sin pétalos maculados (Tabla 1).

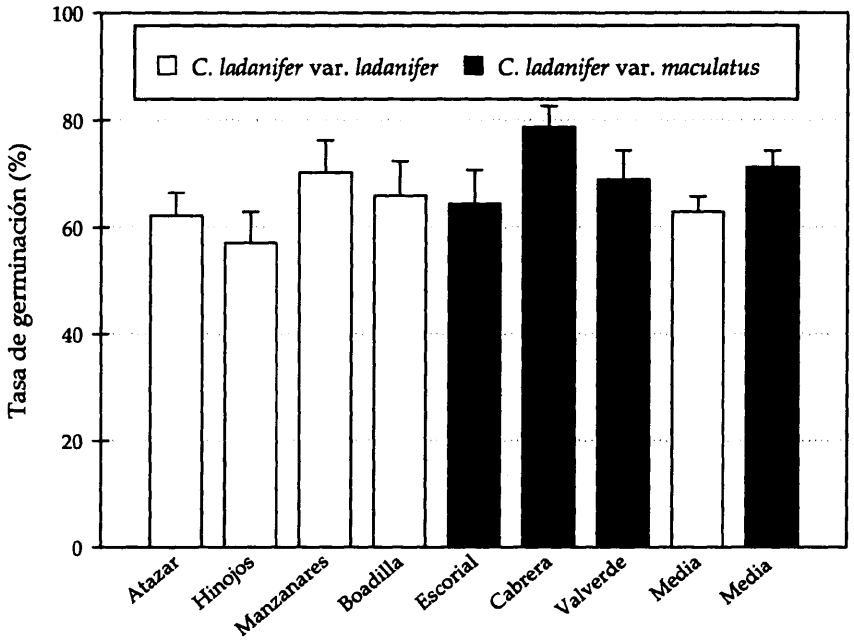

Figura 1. Porcentaje medio de germinación (± intervalos de confianza) de semillas de *Cistus ladanifer* de siete poblaciones de dos variedades distintas (var. ladanifer en blanco y var. maculatus en negro) ($P < 0.05$, estimado mediante remuestreo, 100.000 réplicas). Ver Tabla 1 para resultados estadísticos.

Tabla 1. Resultados del ANCOVA realizado para investigar diferencias en la tasa de germinación de las semillas de *C. ladanifer* en función del individuo, la población y la variedad en siete poblaciones, cinco situadas en la provincia de Madrid (Ata, Cab, Man, Esc, Cha) y dos en la provincia de Huelva (Hin, Val)

	g.l.	SC	*F*	*p*
Individuo (Población*Variedad)	52	17,91	3,40	<0,0001
Población (Variedad)	5	2,90	1,83	0,12
Variedad	1	0,01	0,08	0,77
Peso semillas	1	0,31	3,10	0,08
Variedad*Peso semillas	1	0,07	0,69	0,41
Error	525	53,16		

Delgado *et al.* (2001) encontraron que las semillas más pesadas de la jara pringosa soportan mejor las condiciones de alta temperatura. El hecho de que el fuego y, por tanto, las altas temperaturas, sea uno de los agentes externos encargados de estimular la germinación de las semillas de las jaras (Troumbis & Trabaud 1986) implicaría, por tanto, una mayor tasa de supervivencia y germinación de las semillas más pesadas en condiciones naturales. En principio, nuestros resultados son congruentes con esta afirmación, sin embargo el ligero efecto positivo que ejerce el peso en la tasa de germinación en nuestras poblaciones podría verse reducido en gran medida al tenerse en cuenta no solo la germinación de la semilla sino la supervivencia de la plántula, puesto que tanto factores ambientales como de competencia son decisivos en el establecimiento de los individuos jóvenes. De hecho Delgado *et al.* (2001) encontraron que el peso de las semillas (así como su número por fruto, pues existe un efecto compensatorio entre ambas variables) depende de las condiciones de germinación. Aunque generalmente las semillas más pesadas presentan un mayor *fitness* en determinadas ocasiones es más ventajoso para la planta tener un mayor número de semillas más ligeras que se puedan tener una mayor dispersión. Por otro lado, la variación en la proporción de germinación respecto a factores ambientales y genéticos ha sido estudiada en numerosas especies (Baskin & Baskin 1998). En los ecosistemas Mediterráneos (sumamente variables en el clima, sustrato, relieve y actividades humanas) patrones de germinación que favorezcan el establecimiento de plántulas en diferentes condiciones espaciales y temporales deben ser exitosos (Cruz *et al.* 2003) como así lo demuestra la variación intraindividual y, por tanto, intrapoblacional encontrada en las poblaciones estudiadas de *C. ladanifer* (Tabla 1).

En resumen, sobre la base de nuestros resultados de una tasa de germinación estadísticamente no diferente en poblaciones de las dos variedades de *Cistus ladanifer* (var. *ladanifer, maculatus*) parece lógico pensar que fuerzas selectivas favoreciendo una de ellas no están actuando en aquellas poblaciones donde las dos variedades coexisten.

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Referencias bibliográficas

Baskin CC, Baskin JM (1998) *Seeds: Ecology, biogeography, and evolution of dormancy and germination* Academic Press, San Diego, California, USA.

Corral R, Pita JM, Pérez-García F (1990) Some aspects of seed germination in four species of *Cistus* L. *Seed Science and Technology* **18**, 321-325.

Cruz A, Pérez B, Velasco A, Moreno JM (2003) Variability in seed germination at the interpopulation, intrapopulation and intraindividual levels of the shrub *Erica australis* in response to fire-related cues. *Plant Ecology* **169**, 93-103.

Day RW, Quinn GP (1989) Comparisons of treatments after an analysis of variance in ecology. *Ecological Monographs* **59**, 433-463.

Delgado JA, Serrano JM, López F, Acosta FJ (2001) Heat shock, mass-dependent germination, and seed yield as related components of fitness in *Cistus ladanifer*. *Environmental and Experimental Botany* **46**, 11-20.

Eriksson O (1999) Seed size variation and its effect on germination and seedling performance in the clonal herb *Convallaria majalis*. *Acta Oecologica* **20**, 61-66.

González-Rabanal F, Casal M (1995) Effect of high temperatures and ash on germination of ten species from gorse shrubland. *Vegetatio* **116**, 123-131.

Houssard C, Escarré J (1991) THe effects of seed weight on growth and competitive ability of *Rumex acetosella* from two successional old-fields. *Oecologia* **86**, 236-242.

Howe HF, Richter WM (1982) Effects of seed size on seedling size in *Virola surinamensis*; a within and between tree analysis. *Oecologia* **53**, 347-351.

Keeley JE (1991) Seed germination and life history syndromes in the California Chaparral. *Botanical Review* **57**, 81-116.

Lehtilä K, Syrjänen K (1995) Positive effects of pollination on subsequent size, reproduction, and survival of *Primula veris*. *Ecology* **76**, 1084-1098.

Lindman HR (1974) *Analysis of variance in complex experimental designs* W.H. Freeman & Co, San Francisco.

McGinley MA, Temme DH, Geber MA (1987) Parental investment in offspring in variable environments: theoretical and empirical considerations. *The American Naturalist* **130**, 370-398.

Pérez-García F (1995) Germinación de semillas de *Cistus osbeckiifolius* Webb ex Christ. (Cistaceae). *Botanica Macaronesica* **22**, 25-34.

Pérez-García F (1997) Germination of *Cistus ladanifer* seeds in relation to parent material. *Plant Ecology* **133**, 57-62.

Statsoft (1999) STATISTICA for Windows (Computer Program Manual). In: *Statsoft, Inc.*, Statsoft, Inc., Tucsa.

Troumbis A, Trabaud L (1986) Comparison of reproductive biological attributes of two *Cistus* species. *Acta Oecologica. Oecologia Plantarum* **7**, 235-250.

Winn AA (1988) Ecological and evolutionary consequences of seed size in *Prunella vulgaris*. *Ecology* **69**, 1537-1544.

Zar JH (1999) *Biostatistical analysis* Prentice Hall, Englewood Cliffs, New Jersey.

4

Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae based on ITS, *trnL-trnF*, and *matK* sequences

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Systematics, character evolution, and biogeography of *Cistus* L. (Cistaceae) based on ITS, *trnL-trnF*, and *matK* sequences

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Abstract

This paper presents the first phylogenetic hypotheses for the 20 species of *Cistus* based on plastid (*trnL-trnF*, *matK*) and nuclear (ITS) DNA sequence data. Phylogenetic relationships reveal that: (1) *Halimium* and *Cistus* form a cohesive, natural group; (2) two major lineages of purple-flowered and white-flowered species are defined, except for the purple-flowered *C. parviflorus*; (3) monophyly of conspecific populations is congruent with the circumscription of species. Topological congruence between nuclear and plastid phylogenies does not support a predominant reticulate system of evolution in *Cistus*. Reconstruction of character evolution suggests an increment of number of fruit valves in the Cistaceae from 3 to 12 in a unidirectional manner. In contrast, reproductive characters, such as sepal number, petal color, and style length, evolved multiple times in the course of evolution. A single colonization of *Cistus* into the Canary Islands appears to be responsible for a lineage of four species sharing a most recent common ancestor with five sepals, purple flowers, styles as long as stamens, and five fruit valves. Species diversity in *Cistus* (14) and *Halimium* (8), coupled with sister-group relationships and molecular divergence, lead us to suggest the western Mediterranean as a major center of present-day differentiation, but paleobotanical data indicate an earlier formation of the *Cistus–Halimium* assemblage in different areas.

Keywords: Canary Islands; Character evolution; Cistaceae; *Cistus*; ITS; *matK*; Mediterranean; Systematics; *trnL-trnF*

1. Introduction

Cistus (Cistaceae) is one of the most characteristic genera of the Mediterranean flora. Shrubby species primarily occur as woodland understory and others (*C. ladanifer*, *C. laurifolius*, and *C. monspeliensis*) are dominant in evergreen scrub. The adaptation of the genus to Mediterranean environments is evident from ecological characteristics such as fire-dependent seed germination (Roy and Sonié, 1992; Trabaud and Renard, 1999), insect-dependent pollination (Talavera et al., 1993), flower-dependent reproduction (Herrera, 1987), and spring-dependent phenology (Herrera, 1986). A long history of human activities has favored distribution and abundance of *Cistus* species in the Mediterranean (Thompson, 2005). Impenetrable masses of *Cistus* plants are formed as early successional stages following woodland disturbances such as fire and soil overturning. Co-occurring species of *Cistus* are frequent, particularly in mountain ranges composed by both acidic and basic soils. Environmental specificity referring to substrate confers additional value to acidiphilous and basiphilous species as predictable indicators of woodland disturbances. In marked contrast to the detailed knowledge of ecological characteristics of *Cistus*, understanding of the evolution of morphological characters and phylogenetic relationships within the genus is extremely limited.

Cistaceae comprises about 180 species, typically displaying loculicidal capsules of three valves, except in *Cistus* that is characterized by capsules with five or more valves. Circumscription of species in the eight genera of the Cistaceae is still problematic, particularly in genera

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such as *Helianthemum* and *Halimium*. This has resulted in the publication of multiple combinations for the same taxon under different generic names (Arrington and Kubitzki, 2003). The taxonomy of *Cistus* has traditionally been based on vegetative (nerve number, shape, and hairiness of leaves) and reproductive characters (sepal number, petal color, style length, and number of fruit valves). Worldwide monographs of *Cistus* have recognized between 16 species (Grosser, 1903) and 28 species (Dunal, 1824) (Table 1). Following Grosser's (1903) treatment with additional species described more recently, the genus is currently thought to comprise approximately 20 species, of which 16 occur in Europe (Warburg, 1968), 11 in Spain (Martín and Guinea, 1949), 12 in Iberia (Demoly and Montserrat, 1993), and 12 in Morocco (Soriano, 2002) (Fig. 1). The highest species diversity therefore occurs in the western Mediterranean, where 14 species are distributed in the Iberian Peninsula and northwestern Africa.

Disparate infrageneric classifications of *Cistus* have been proposed (Table 1). In the last taxonomic treatment three subgenera, namely *Cistus*, *Leucocistus*, and *Halimioides*, are described based on morphological characters (Demoly and Montserrat, 1993). The subgenus *Halimioides* (three species) is distributed exclusively in the western Mediterranean, whilst the subgenera *Leucocistus* (eight species) and *Cistus* (nine species) occur in the Mediterranean basin and the Canary Islands. This widespread distribution of *Cistus* subgenera and species clearly indicates the successful mobility of seeds and colonization in Mediterranean habitats.

Evolutionary mechanisms responsible for the morphological diversity within *Cistus* remain poorly understood. Plants are predominantly self-incompatible (Bosch, 1992) promoting crossing between individuals of the same and different species. Identification in the field of individuals as hybrids is relatively easy because they display characteristics that are intermediate between those of nearby, putative progenitors. Crossing between two plants of any species potentially generates offspring with intermediate traits, particularly when they are closely related congeners (Demoly, 1996). Hybrid polyploidy (allopolyploidy) has not played an important role in speciation of *Cistus*, as all species display a chromosome number of $2n = 18$. In fact, variation of DNA content is not significant among species (Ellul et al., 2002).

A proper phylogeny of *Cistus* has not been proposed to date. Dansereau (1939) outlined a phyletic diagram based on morphological features. Examination of 13 isozyme loci indicates high values of genetic divergence among four Canarian species (Batista et al., 2001), although evolutionary relationships of island endemics with respect to continental species remain unknown. Phylogenetic relationships among Cistaceae genera indicate that *Cistus* is closely related to *Halimium* and *Helianthemum* (Arrington and Kubitzki, 2003; Savolainen et al., 2000). In addition, angiosperm phylogenies reveal that the family forms a lineage coupled with Dipterocarpaceae and Sarcolaenaceae (Soltis et al., 2000). A larger sample is needed, however, to determine sister-group relationships of Cistaceae and *Cistus*.

Four basic objectives are addressed in the present study: (1) to evaluate congruence between nuclear (ITS) and plastid (*trnL-trnF*, *matK*) sequences; (2) to identify major lineages and test the monophyly of infrageneric groupings recognized in existing classifications of *Cistus*; (3) to interpret evolution of key morphological characters; and (4) to describe biogeographic patterns in the Mediterranean basin and in the colonization of the Canary Islands.

2. Materials and methods

2.1. DNA extraction, gene amplification, and sequencing

A total of 47 individuals representing the 20 species of *Cistus*, one of *Fumana*, two of *Halimium*, two of *Helianthemum*, and one of *Tuberaria* were sampled for ITS, *trnL-F*, and *matK* sequencing (Supplementary Table S1). Total genomic DNA was extracted from material collected in the field, material in the living collections of R.G. Page, O. Filippi, and the Royal Botanic Garden of Madrid, and from two herbarium specimens (MA). Field collections were dried in silica gel. DNA was extracted using Kneasy Plant Mini Kit (Qiagen, California) and amplified using the polymerase chain reaction (PCR) on a Perkin-Elmer PCR System 9700 (California) or an MJ Research (Massachusetts) thermal cycler. After 1–4 min pretreatment at 94 °C, PCR conditions were: 24–35 cycles of 1 min at 94 °C, 30 s–1 min at 50–52 °C, and 1–2 min at 72 °C. Standard primers were used for amplification of the *trnL*(UAA)-*trnF*(GAA) spacer (Taberlet et al., 1991), the *matK* intron (Johnson and Soltis, 1994), and the ITS region (Sun et al., 1994 for 17SE; White et al., 1990 for ITS4). A volume of 1 μL of dimethyl sulfoxide (DMSO) was included in each 25 μL reaction. Amplified products were cleaned using spin filter columns (PCR Clean-up kit, MoBio Laboratories, California) following the manufacturer's protocols. Cleaned products were then directly sequenced using dye terminators (Big Dye Terminator v. 2.0, Applied Biosystems, Little Chalfont, UK) following the manufacturer's protocols and run into polyacrylamide electrophoresis gels (7%) using an Applied Biosystems Prism Model 3700 automated sequencer. PCR primers were used for cycle sequencing of the *trnL-F* spacer and the *matK* intron, while the ITS5 and ITS4 (Sun et al., 1994) primers were used for cycle sequencing the ITS region. Sequenced data were assembled and edited using the program Seqed (Applied Biosystems, California). The limits of the

Table 1
Comparison of historical taxonomic treatments of *Cistus* using taxa names as published in original publications

Dunal (1824)	Spach (1836)	Willkomm (1856)
Sect. I. *Erythrocistus* Dunal	Genus *Ladanium* Spach	Subgen. I. *Erythrocistus* Dunal
C. albidus L.	*L. officinarum* Spach (*C. ladanifer* L.)	Sect. I. *Macrostylia* Willk.
C. candidissimus Dunal (*C. ochreatus* C. Sm. ex Buch)	*L. laurifolium* Spach (*C. laurifolius* L.)	*C. vaginatus* Aiton (*C. symphytifolius* Lam.)
C. complicatus Lam. (*C. parviflorus* Lam.)	*L. cyprium* Spach (*C. ladanifer* x *C. laurifolius*)	*C. candidissimus* Dunal (*C. ochreatus* C. Sm. ex Buch)
C. creticus L.		Sect. II. *Brachystylia* Willk.
C. crispus L.	Genus *Rhodocistus* Spach	*C. albidus* L.
C. cymosus Dunal (*C. parviflorus* x *C. creticus*)	*R. berthelotianus* Spach (*C. symphytifolius* Lam.)	*C. polymorphus* Willk. (*C. creticus* L.)
C. heterophyllus Desf.		*C. creticus* L.
C. hybridus Vahl (?)	Genus *Cistus* (Tourn.) Spach	*C. crispus* L.
C. incanus L. (*C. albidus* x *C. crispus*)	Sect. I. *Rhodopsis* Spach	*C. heterophyllus* Desf.
C. parviflorus Lam.	*C. purpureus* Lam. (*C. ladanifer* x *C. creticus*)	*C. purpureus* Lam. (*C. ladanifer* x *C. creticus*)
C. purpureus Lam. (*C. ladanifer* x *C. creticus*)	Sect. II. *Eucistus* Spach	Sect. III. *Astylia* Willk.
C. sericeus Vahl (*C. albidus* ?)	*C. vulgaris* Spach (*C. creticus* L.)	*C. parviflorus* Lam.
C. undulatus Dunal (*C. creticus* L.)	Sect. III. *Ledonella* Spach	
C. vaginatus Dryand. (*C. symphytifolius* Lam.)	*C. parviflorus* Spach (*C. parviflorus* Lam.)	Subgen. II. *Leucocistus* Willk.
C. villosus Lam. (*C. creticus* L.)		Sect. IV. *Stephanocarpus* Spach
	Genus *Stephanocarpus* Spach	*C. monspeliensis* L.
Sect. II. *Ledonia* Dunal	*S. monspeliensis* Spach (*C. monspeliensis* L.)	*C. pouzolzii* Delile
C. clusii Dunal		*C. florentinus* Lam. (*C. monspeliensis* x *C. salviifolius*)
C. corbariensis Pourr. (*C. populifolius* x *C. salviifolius*)	Genus *Ledonia* Spach	Sect. V. *Ledonia* Spach
C. cyprius Lam. (*C. ladanifer* x *C. laurifolius*)	*L. heterophylla* Spach (*C. monspeliensis* x *C. populifolius*)	*C. ledon* Lam. (*C. laurifolius* x *C. monspeliensis*)
C. florentinus Lam. (*C. monspeliensis* x *C. salviifolius*)	*L. populifolia* Spach (*C. populifolius* L.)	*C. populifolius* L.
C. hirsutus Lam. (*C. psilosepalus* Sweet)	*L. hirsuta* Spach (*C. psilosepalus* Sweet)	*C. longifolius* Lam. (*C. monspeliensis* x *C. populifolius*)
C. ladaniferus L. (*C. ladanifer* L.)	*L. peduncularis* Spach (*C. salviifolius* L.)	*C. obtusifolius* Sweet (*C. psilosepalus* x *C. salviifolius*)
C. laurifolius L.		*C. hirsutus* Lam. (*C. psilosepalus* Sweet)
C. laxus Aiton (*C. populifolius* x *C. psilosepalus* ?)		*C. salviifolius* L.
C. ledon Lam. (*C. laurifolius* x *C. monspeliensis*)		Sect. VI. *Ladanium* Spach
C. longifolius Lam. (*C. monspeliensis* x *C. populifolius*)		*C. cyprius* Lam. (*C. ladanifer* x *C. laurifolius*)
C. monspeliensis L.		*C. ladaniferus* L. (*C. ladanifer* L.)
C. populifolius L.		*C. laurifolius* L.
C. salviifolius L.		Sect. VII. *Halimioides* Willk.
		C. clusii Dunal
		C. bourgaeanus Coss. (*C. libanotis* L.)
		C. sericeus Munbyi (*C. munbyi* Pomel)

Grosser (1903)	Dansereau (1939)	Demoly and Montserrat (1993) (Iberian species)
Group A.	Subgen. I. *Erythrocistus* (Dunal) Willk.	Subgen. I. *Cistus* L.
Sect. I. *Rhodocistus* (Spach) Grosser	Sect. I. *Macrostylia* Willk.	*C. albidus* L.
C. ochreatus C. Sm. ex Buch	*C. osbeckiaefolius* Webb ex Christ (*C. osbeckiifolius* Webb ex Christ)	*C. creticus* L.
C. symphytifolius Lam.	*C. symphytifolius* Lam.	*C. crispus* L.
Sect. II. *Eucistus* Spach		*C. heterophyllus* Desf.
C. albidus L.	Sect. II. *Erythrocistus* Dunal	
C. villosus L. (*C. creticus* L.)	*C. albidus* L.	Subgen. II. *Leucocistus* Willk.
C. crispus L.	*C. villosus* L. (*C. creticus* L.)	Sect. 1. *Ledonia* Dunal
C. heterophyllus Desf.	*C. crispus* L.	*C. monspeliensis* L.
Sect. III. *Ledonella* Spach	*C. heterophyllus* Desf.	*C. populifolius* L.
C. parviflorus Lam.x	Sect. III. *Ledonella* Spach	*C. psilosepalus* Sweet
	C. parviflorus Lam.	*C. salviifolius* L.
Group B.		Sect. 2. *Ladanium* (Spach) Gren.
Sect. IV. *Stephanocarpus* (Spach) Willk.	Subgen. II. *Leucocistus* Willk.	*C. ladanifer* L.
C. monspeliensis L.	Sect. IV. Stephanocarpoidea Rouy et Foucaud	*C. laurifolius* L.
Sect. V. *Ledonia* Dunal	*C. varius* Pourr. (*C. pouzolzii* Del.)	
C. populifolius L.	Sect. V. *Stephanocarpus* (Spach) Gren.	Subgen. III. *Halimioides* (Willk.) Demoly & P. Monts.
C. hirsutus Lam. (*C. psilosepalus* Sweet)	*C. monspeliensis* L.	*C. clusii* Dunal
C. salviifolius L.	Sect. VI. *Ledonia* Dunal	*C. libanotis* L.
	C. populifolius L.	
Group C.	*C. hirsutus* Lam. (*C. psilosepalus* Sweet)	
Sect. VI. *Ladanium* (Spach) Willk.	*C. salviifolius* L.	
C. ladaniferus L. (*C. ladanifer* L.)	Sect. VII. *Ladanium* (Spach) Gren. et Godr.	
C. laurifolius L.	*C. ladaniferus* L. (*C. ladanifer* L.)	
Sect. VII. *Halimioides* Willk.	*C. laurifolius* L.	
C. rosmarinifolius Pourr. (*C. clusii* Dunal)	Sect. VIII. *Halimioides* Willk.	
C. bourgaeanus Coss. (*C. libanotis* L.)	*C. libanotis* L. (*C. clusii* Dunal)	
C. sericeus Munby (*C. munbyi* Pomel)	*C. bourgeanus* Coss. (*C. libanotis* L.)	
	C. munbyi Pomel	

Taxa in brackets as interpreted.

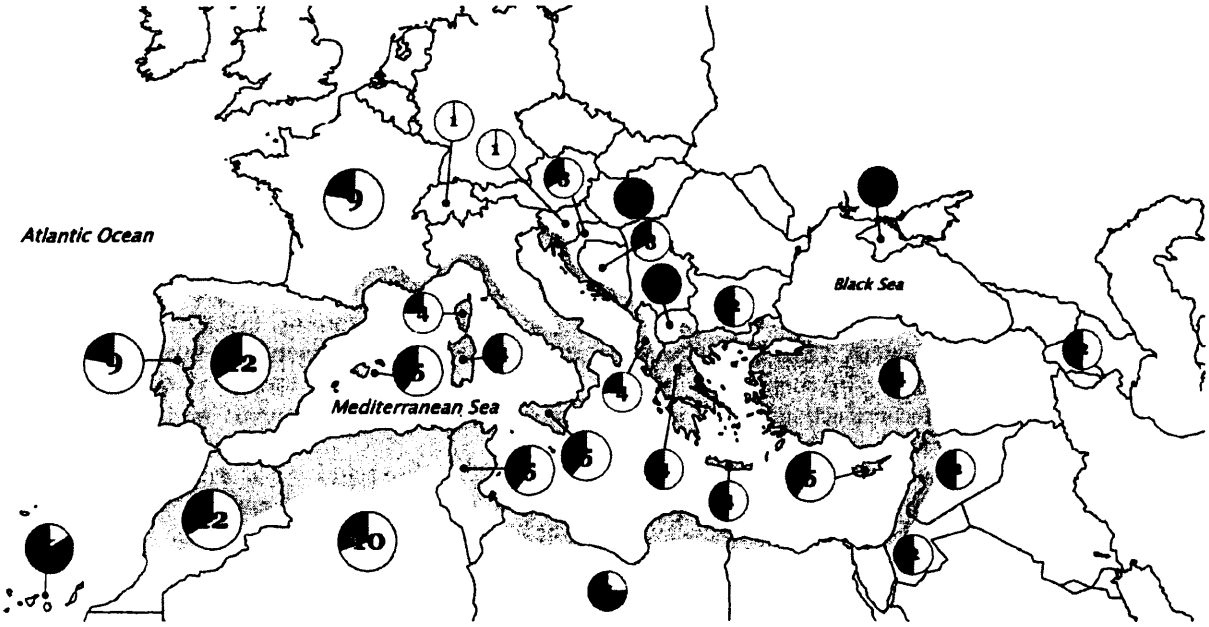

Fig. 1. Distribution map and number of *Cistus* species. Pie diagrams include proportion of white-flowered (white) and purple-flowered (grey) species in every country. Notice the highest species diversity in the western Mediterranean. The Mediterranean region shown in grey.

regions were determined by position of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

2.2. Molecular analysis

Phylogenetic analyses were performed on three molecular data sets (*trnL-F*, *matK*, and ITS) using the same methodology and a similar number of sequences from each of the 20 species of *Cistus* and six of Cistaceae (Supplementary Table S1). In addition, an analysis of *trnL-F* sequences from Old World Cistaceae (*Fumana*, *Helianthemum*, *Tuberaria*, *Halimium*, and *Cistus*) and Dipterocarpaceae was performed to investigate relationships of *Cistus* with respect to another Cistaceae. In this analysis, four Dipterocarpaceae (*Dipterocarpus*, *Parashorea*, *Shorea*, and *Hopea*) from GenBank (Li et al., unpublished) were used as outgroup taxa on the basis of an earlier *rbcL* phylogeny (Ducousso et al., 2004).

Sequences were aligned using Clustal X 1.62b (Thompson et al., 1997), with further adjustments by visual inspection. Insertion/deletion mutations (indels) were manually coded for parsimony analyses as appended characters following the logic of Kelchner (2000) and Simmons and Ochoterena (2000). Maximum Parsimony (MP) and Bayesian Inference (BI) analyses were then performed on each data set. All parsimony analyses were conducted using Fitch parsimony (as implemented in PAUP*; Swofford, 1999) with equal weighting of all characters and of transitions/transversions. Heuristic searches were replicated 100 times with random taxon-addition sequences, tree-bisection-reconnection (TBR) branch swapping, and the options MulTrees and Steepest Descent in effect. Additionally, as a result of memory limitation in completing the analysis of *trnL-F* sequences of the Dipterocarpaceae–Cistaceae matrix, 10 trees only were saved from each of the 1000 replicates to minimize time searching thousands of trees. All trees thus collected were combined and used as starting trees, with MulTrees on and no tree limit (these trees were then swapped to completion) and Subtree–Pruning-Regrafting (SPR) (Salamin et al., 2003). Internal support was assessed using 1000 replicates with simple taxon addition and SPR branch swapping, but permitting only 10 trees per replicate to be held (Chase et al., 2003).

To determine the simplest model of sequence evolution that best fits the sequence data, the Hierarchical Likelihood Ratio Test (hLRT) and Akaike Information Criterion (AIC) were implemented using MrModeltest 1.1b (Posada and Crandall, 1998; Nylander, 2002). A Bayesian Inference analysis was conducted on each data set using MrBayes 3.0b4 (Ronquist and Huelsenbeck, 2003) and sampling for one million generations (four MCMC, chain temperature = 0.2; sample frequency = 100; and burn-in <500). A 50% majority-rule consensus tree was calculated for each matrix from the pooled sample using the *sumt* command to yield the final Bayesian estimate of phylogeny. We used posterior probability (PP) as alternative estimate of robustness (Alfaro et al., 2003).

To assess whether data provide significantly less support for a specified alternative topology, we used the Significantly Less Parsimonious test of Templeton (SLP_T)

(Templeton, 1983) and compared matrices and most parsimonious topologies recovered by analyzing ITS, *trnL-F*, and *matK* sequences. SLP_T was implemented in PAUP* using the strict consensus tree obtained from parsimony analyses of the data (Johnson and Soltis, 1998).

2.3. Morphological characters

The distribution of 10 morphological characters, upon which classification of *Cistus* has been traditionally based, is indicated in Supplementary Table S2. Information on some characters is missing for some species and we consequently performed reconstructions of two vegetative (shape and base of leaves) and four reproductive (sepal and fruit-valve number, petal color, style length) characters. Patterns of evolution were explored using the character-state optimization function of MacClade 4.06 (Maddison and Maddison, 1992), assuming Fitch parsimony. Both ACCTRAN (maximizing the proportion of the homoplasy that is accounted by parallelism) and DELTRAN (maximizing the proportion accounted by reversal) optimizations were considered and analyzed. Characters were traced initially onto the strict consensus of shortest trees obtained. To gain further insights into morphological character evolution, the MP tree displaying most congruence with the BI tree, under the simplest model of sequence evolution, was chosen (see below).

3. Results

3.1. Characteristics of trnL-F, ITS, and matK sequences

The characteristics of the three data sets are summarized in Table 2. Within *Cistus*, *trnL-F* sequence divergence ranges from 0.0% (between the 17 conspecific accessions and between *C. clusii–C. munbyi*, *C. symphytifolius–C. chinamadensis*, and *C. albidus–C. creticus*) to 3.15% (between *C. parviflorus* 1–*C. monspeliensis* 1) using the K-2-p model of evolution; *matK* sequence divergence ranges from 0.0% (between 12 conspecific accessions and between *C. albidus–C. creticus*, *C. albidus–C. heterophyllus*, and *C. creticus–C. heterophyllus*) and 1.78% (between *C. salviifolius–C. osbeckiifolius*); and ITS sequence divergence ranges from 0.0% (between eight conspecific accessions) to 4.86% (between *C. crispus–C. parviflorus*). Nucleotide additivity for direct ITS sequencing was clearly observed in direct and reverse chromatograms at 15 positions of 10 accessions (see Supplementary table S2). More than one ITS copy with different sequence length was also detected in two accessions (*C. psilosepalus* 1, *C. parviflorus* 1). A single gap allowed re-establishing nucleotide chromatogram matching, and the resulting sequence was used in the phylogenetic analyses.

3.2. Phylogenetic analyses

Availability (Li et al., in GenBank) and alignability (clustal X, Thompson et al., 1997) of *trnL-F* sequences using four Dipterocarpaceae genera (*Dipterocarpus*, *Parashorea*, *Shorea*, and *Hopea*) allowed performing suitable phylogenetic analysis of Cistaceae–Dipterocarpaceae accessions. MP and BI analyses recognize Cistaceae as monophyletic, with 100% bootstrap value (BS) and 100 posterior probability (PP). The strict consensus tree of 362,200 MP trees is shown in Fig. 2. Within Cistaceae, a successive branching is depicted in the strict consensus tree, in which *Fumana* comes out first (92% BS) followed by *Helianthemum* (100% BS), and then *Tuberaria*. Accessions of *Halimium* and *Cistus* form a largely

Table 2
Summary of phylogenetic characteristics obtained from the analyses of ITS, *trnL-trnF*, and *matK* sequences of the Cistaceae and *Cistus*

	ITS				*trnL-trnF*	*matK*
	ITS region	ITS-1	5.8 S	ITS-2		
Cistaceae						
Length range (bp)	585–671	201–268	167	199–248	377–461	1302–1357
Aligned length (bp)	698	274	168	256	505	1403
Number of variables/informative characters	203/104	108/62	4/3	91/39	127/66	265/143
Maximum sequence divergence (K-2-p)	20.03%	29.70%	2.47%	33.86%	20.07%	13.75%
Informative indels (no. bp)	19 (1–41)	9 (1–41)	0	10 (1–29)	15 (1–26)	17 (1–48)
CI′ (CI)	0.64 (0.78)	—	—	—	0.84 (0.9)	0.87 (0.92)
RI	0.82	—	—	—	0.93	0.95
Mean G + C content	65%	69%	49%	68%	33%	33%
Cistus						
Number of variables/informative characters	92/73	58/45	1/0	33/28	48/42	56/46
Maximum sequence divergence (K-2-p)	4.86%	9.33%	0.60%	5.26%	3.15%	1.78%
Informative indels (no. bp)	7 (1–2)	4 (1–2)	0	3 (1–2)	10 (1–26)	3 (1–48)
Number of nucleotide additivities	15	10	0	5	0	0
Number of accessions with nucleotide additivities	10	7	0	7	0	0

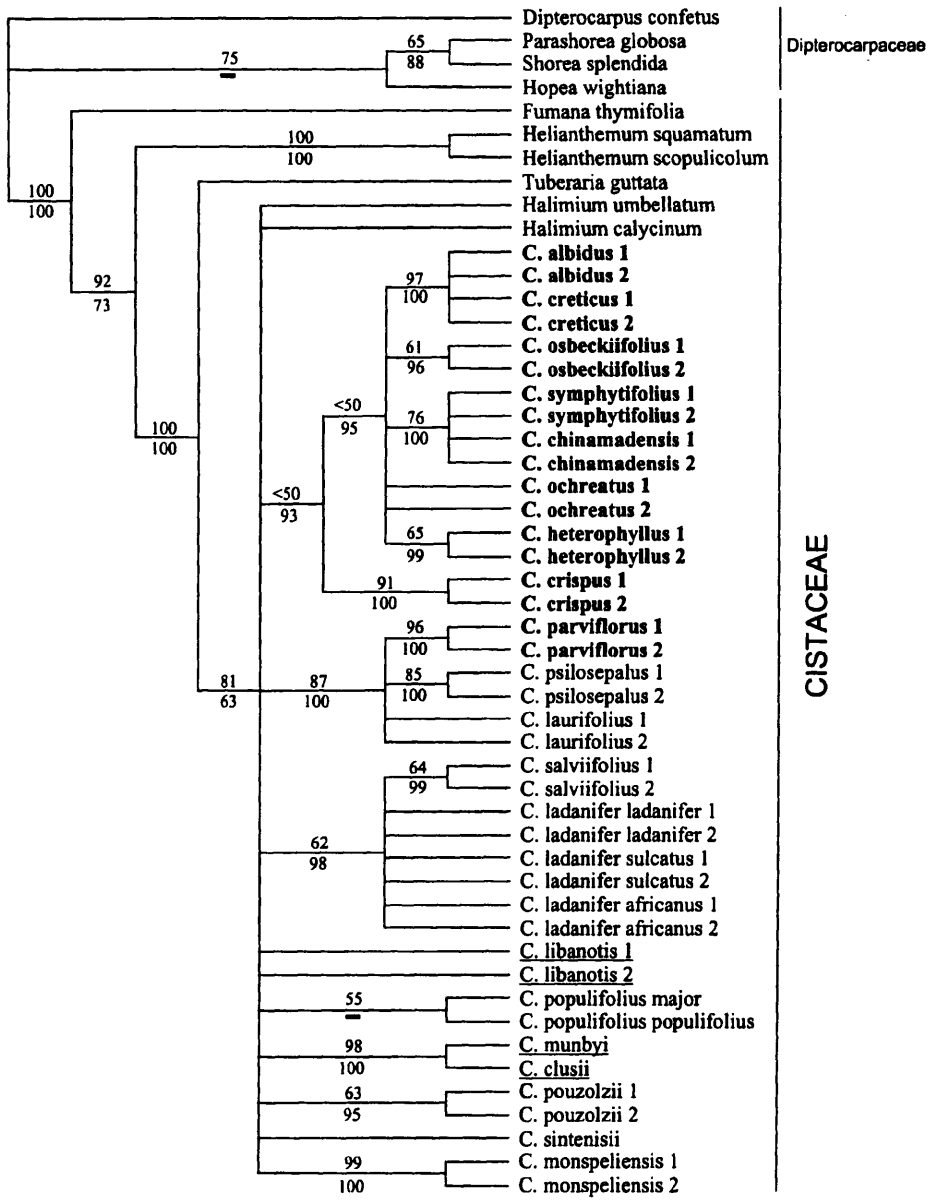

Fig. 2. Strict consensus tree of 362,200 shortest trees of 220 steps (CI = 0.90; CI′ = 0.83, excluding uninformative characters; RI = 0.94) from the analysis of *trnL-F* sequences. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR + *G* as the simplest model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen (-) below branches. Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

unresolved, monophyletic group (81% BS). Bayesian inference, using GTR + *G* as the simplest model of sequence evolution, reached equilibrium after 350,000 generations. The BI reconstruction is mostly consistent with the strict consensus tree, but more resolved: (i) *Halimium calycinum* is sister to *Cistus* (76 PP), whilst *Halimium umbellatum* is nested within a group of white-flowered *Cistus* species (67 PP); (ii) within this group, a subgroup of six species (*C. laurifolius*, *C. parviflorus*, *C. psilosepalus*, *C. pouzolzii*, *C. populifolius*, and *C. monspeliensis*) is also retrieved (80 PP); and (iii) *C. monspeliensis* and *C. populifolius* are sister species (95 PP) (results not shown). In the MP and BI analyses, accessions of the same species either formed monophyletic groups or were placed in unresolved polytomies. We used hereafter *Fumana thymifolia* as the outgroup taxon based on its sister-group relationship to the rest of Cistaceae in the *trnL-F* (Fig. 2) and *rbcL* (Guzmán et al., unpublished) analyses. The analysis of Cistaceae-only accessions using *Fumana* as the outgroup taxon resulted in a

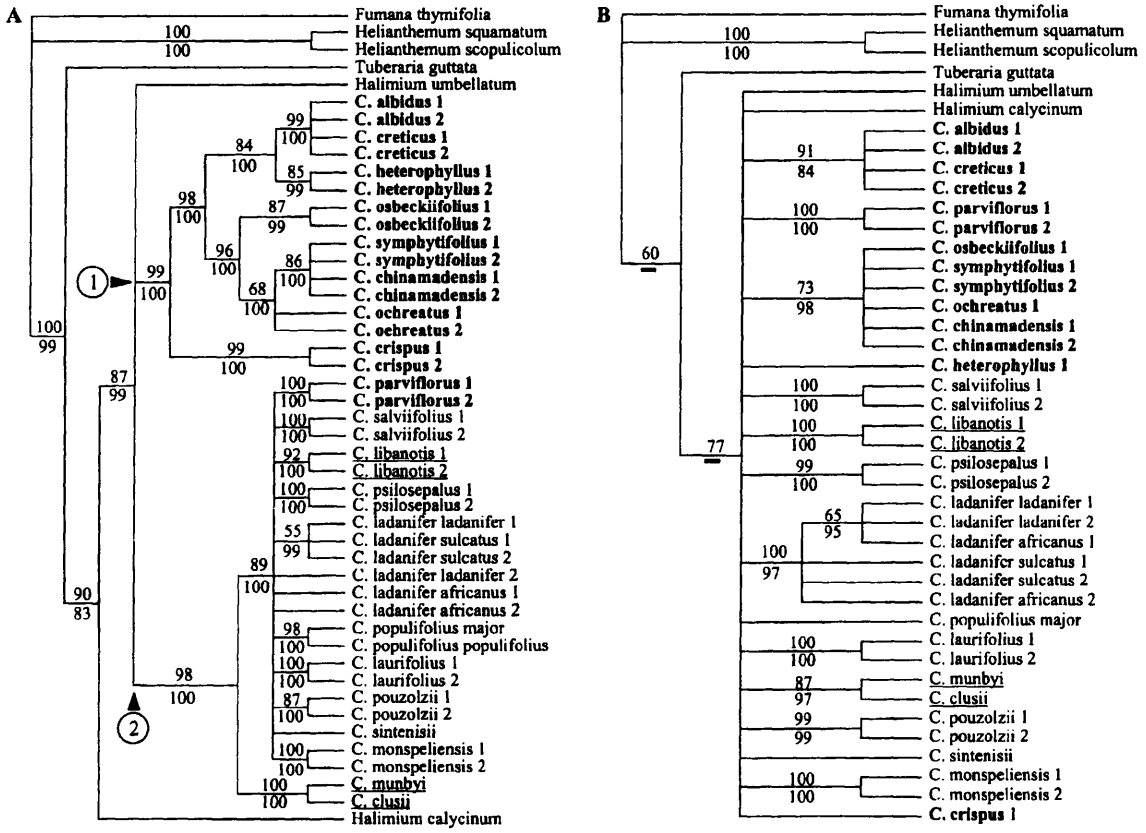

Fig. 3. Strict consensus trees of MP analyses. *F. thymifolia* served as the outgroup taxon. Insertions/deletions (indels) recorded as additional characters. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities from the Bayesian analysis under the GTR + *G* model of DNA substitution selected by Modeltest 3.06 (Posada and Crandall, 1998). BI resolution incongruent with MP clades as indicated with a hyphen below branches (-). Two major clades indicated in circled numbers. (A) Strict consensus tree of 224 shortest trees of 512 steps (CI = 0.90; CI′ = 0.85 excluding uninformative characters; RI = 0.94) from the combined analysis of *trnL-F* and *matK* sequences. (B) Strict consensus tree of 69,486 most parsimonious trees of 933 steps (CI = 0.78; CI′ = 0.64; RI = 0.82) from the analysis of ITS-region sequences. Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

similar resolution, support, and consistency indices (see Table 2).

The GTR + *G* model was also selected for the *matK* data set. MP and BI analyses yielded similar topology, although with less resolution and lower support values in the MP analysis (results not shown). The strict consensus tree of the combined *matK* and *trnL-F* sequences depicts *Halimium* and *Cistus* as monophyletic (90% BS), and a basal polytomy is formed by *H. umbellatum* and two *Cistus* clades (Fig. 3A). A well-resolved *Cistus* clade (clade 1) comprises all purple-flowered species (99% BS) except for *C. parviflorus*. A second *Cistus* clade (clade 2) consists of all white-flowered species plus *C. parviflorus* (98% BS). The BI analysis of the combined *matK* and *trnL-F* matrix using the common, simplest model of sequence evolution for both data sets (GTR + *G*) reached equilibrium after 40,000 generations. Again, the BI analysis displays better resolution and higher support values than those of the MP analysis, including the sister relationship of *H. calycinum* to a group of all *Cistus* species and *H. umbellatum* and of this *Halimium* species to the group of white-flowered *Cistus* species (clade 2). High BS and PP values (over 85 support values) were retrieved for 11 groups of conspecific accessions (Fig. 3A).

The analysis of ITS sequences yielded limited resolution (Fig. 3B). Eight conspecific accessions are resolved into well-defined monophyletic groups, mostly in agreement with those in the plastid DNA tree (Fig. 3A). In addition, other supported clades are: the four accessions of *C. albidus–C. creticus* (91% BS); the six Canarian accessions (73% BS); and the two accessions of *C. clusii–C. munbyi* (87% BS). Bayesian inference using the selected GTR + *G* + *I* model reached equilibrium after 50,000 generations. The BI analysis retrieved similar relationships at clade tips to those in Fig. 3B, plus a group of *C. psilosepalus* sister to *C. ladanifer* accessions (88 PP) and *C. heterophyllus* sister to the Canarian group (82 PP). Visual inspection of ITS chromatograms revealed 15 positions containing nucleotide double

peaks (Table 2). Although it was not possible to determine whether, in some cases, double-peak patterns may be the result of sequencing artifacts, equimolar proportions of alternative nucleotide peaks in many accessions suggested the presence of more than one ITS copy. This view is supported by the facts that forward and reverse chromatograms displayed double peaks of the same nucleotide proportions and that five affected matrix positions turned to be parsimony-informative characters.

In the analysis of the Cistaceae, resolution and support at clade tips and deep nodes is higher in plastid than in nuclear trees. Consensus-tree topologies display polytomies primarily as a result of insufficient number of informative characters and character incongruence across accessions. In *Cistus*, the number of parsimony-informative characters is higher in the ITS (73) than in the *trnL-F* (42) and *matK* (46) sequences, indicating that the ITS analysis had a sufficient number of informative characters for better resolution. A search for the causes behind low levels of resolution revealed higher measure of fit for the *trnL-F* and *matK* analyses ($CI' = 0.84$ and $CI' = 0.87$, respectively) than that for the ITS analysis (0.64). These values fall into the CI and RI range provided by Álvarez and Wendel (2003), who also reported that ITS data sets have higher levels of homoplasy in angiosperms than plastid data sets. Additionally, the occurrence of more than one nucleotide (additivity) at the same seven informative sites in some ITS accessions contributed to a low resolution, as a result of multiple searches using alternative character states.

3.3. Data congruence and combined phylogeny

The plastid genome is generally considered free from recombination and the *trnL-F* and *matK* sequences consequently share a hypothetical common phylogenetic history. This provides a strong argument for inferring character evolution by combining *a priori* both data sets. Additionally, a significance test for heterogeneity between nuclear and plastid data sets was implemented. Characters in each of the three data sets statistically support alternative topologies found in the set of the shortest trees recovered for those data sets (Table 3). As statistical sub-optimality exists in at least one direction in each comparison, the SLP_T supports data homogeneity (Johnson and Soltis, 1998). The combined plastid and nuclear data matrix of 42 samples consisted of 2606 characters, of which the number of variable/parsimony-informative characters was 595/313 in the Cistaceae (Table 2). The strict consensus tree reveals, once again, a well-defined assemblage of all the *Halimium* and *Cistus* accessions (99% BS), in which *H. calycinum* is sister (71% BS) to a group formed by *H. umbellatum* and two clades of *Cistus* (Fig. 4). Clade 1 contains exclusively purple-flowered, 5-sepaled, mid-to-long styled species (subgenus *Cistus*) (100% BS). Within clade 1, *C. crispus* is sister (96% BS) to the remaining members; they, in turn, form three subclades. The first is a well-defined (100% BS) group of *C. albidus* and *C. creticus* accessions. The second forms a well-supported group (98% BS) of Canarian species, and includes a subgroup of *C. symphytifolius* and *C. chinamadensis* accessions (95% BS). *C. heterophyllus* constitutes the third, unresolved subclade. Clade 2 contains all white-flowered species of subgenera Leucocistus and Halimioides, plus the purple-flowered *C. parviflorus* (93% BS). The three species of subgenus Halimioides do not form a monophyletic group. Whilst *C. munbyi* and *C. clusii* constitute a clade (100% BS) that is resolved as sister to the rest of species in clade 2, *C. libanotis* is unresolved in the large polytomy of white-flowered species. The results from the BI analysis, implementing partitions with the respective simplest models of evolution, were consistent with the strict consensus of the MP trees, but with better resolution and similar or higher support values in most cases. Interestingly, a group of white-flowered *Cistus–Halimium* species is resolved in the BI tree (96 PP), displaying a pectinate topology and high support values (results not shown). In this BI tree, *H. umbellatum* is sister to the white-flowered species of *Cistus* (100 PP), which are also arranged in a pectinate fashion with *C. munbyi–C. clusii* (99 PP) as the earliest diverging group, followed by *C. libanotis* (86 PP), *C. sintenisii* (56 PP), and then a biphyletic group consisting of *C. salviifolius* sister to *C. ladanifer* (97 PP) and *C. monspeliensis* sister to the remaining five species (99 PP) (results not shown). Multiple conspecific accessions within clade 2 form well-supported monophyletic groups (100% BS, 100 PP) in eight cases (*C. parviflorus*, *C. salviifolius*, *C. libanotis*, *C. psilosepalus*, *C. ladanifer*, *C. laurifolius*, *C. pouzolzii*, and *C. monspeliensis*).

Table 3
Results of the Templeton's test (SLP_T) on alternative topologies of strict consensus trees

Data set	Alternative topology	Increase	Decrease	Net	Probability
ITS	*trn*L-F	28	8	48	0.028*
*trn*L-F	ITS	34	2	36	0.0001*
ITS	*mat*K	27	4	50	0.825
*mat*K	ITS	58	49	67	0.0001*
*trn*L-F	*mat*K	18	13	23	0.24
*mat*K	*trn*L-F	19	1	28	0.0021*

Probability values greater than 0.05 indicate that the alternative topology is not significantly less parsimonious than at least one shortest tree.

3.4. Character-state reconstruction

A summary of significant character states obtained from the literature and from our own observations is shown in Fig. 4. Exploration of character changes and ancestral-state reconstruction was undertaken using the total-evidence analysis of nuclear and plastid sequences.

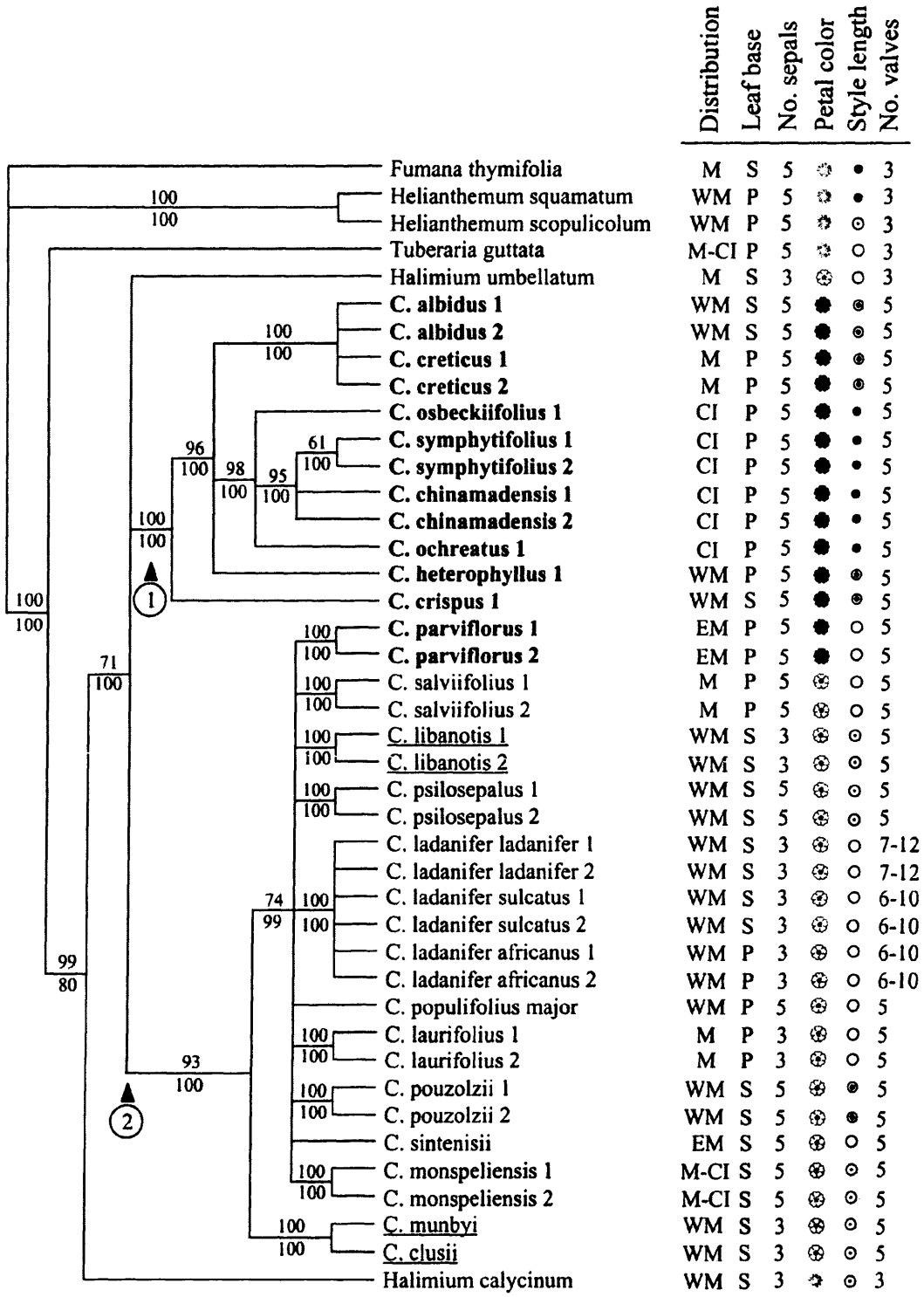

Fig. 4. Strict consensus tree of 328 shortest trees of 895 steps (CI = 0.81; CI′ = 0.70; RI = 0.85) from the combined analysis of *trnL-F*, *matK*, and ITS sequences (total-evidence tree). Insertions/deletions (indels) recorded as additional characters. *F. thymifolia* served as the outgroup taxon. Numbers above branches are bootstrap values. Numbers below branches show posterior probabilities. Species distribution (M, Mediterranean; WM, western Mediterranean; EM, eastern Mediterranean; and CI, Canary Islands) and five relevant morphological characters are plotted on the right side of the tree: leaf base (P, petiolate; S, sessile), number of sepals (3, 5); petal color (◌, yellow; ⊛, white; and ●, purple); style length (○, sessile; ⊙, shorter than stamens; ◉, as long as stamens; and ●, longer than stamens); and number of fruit valves (3, 5, 6 or more). Taxa circumscription in subgenera is coded as follows: Cistus (in bold); Leucocistus (in roman), and Halimioides (underlined).

ACCTRAN and DELTRAN optimizations gave extremely similar results, and only results using ACCTRAN are presented. The most likely of the shortest trees was chosen based on congruence with the BI analysis under the simplest model of sequence evolution (Fig. 5). MacClade reconstructions of character states indicate that leaf shape, sepal number, petal color, and style length are homoplastic in the *Cistus–Halimium* assemblage. For example, purple petals seem to have occurred twice, being the only flower color maintained in the eight species of clade 1. Trace of the four states for style length (sessile, shorter, similar, and longer than stamens) appears to be extremely complex, arising many times not only in this assemblage, but also in the Cistaceae (Fig. 5A). The number of fruit valves is not a synapomorphy supporting the monophyly of *Cistus* (which has five or more valves), in contrast to the three valves found in the remaining seven genera of Cistaceae. Remarkably, the three subspecies of *C. ladanifer* do not display five valves. Rather, between 6 and 12 fruit divisions are observed within this species, representing a unique increment in fruit segmentation from a 3-valved ancestor (Fig. 5B).

4. Discussion

4.1. Systematic implications

Analysis of *trnL-F* sequences supports the monophyly of the Cistaceae genera using *Fumana*, *Helianthemum*, *Tuberaria*, *Halimium*, and *Cistus* (Fig. 2). All phylogenetic analyses are congruent with the monophyly of the *Cistus–Halimium* assemblage. A close relationship between these two genera was suggested in a phyletic diagram by Dansereau (1939). The two representatives of *Halimium* section Halimium (*H. umbellatum*) and section Commutata (*H. calycinum*) appear to have arisen from the same lineage involved in the formation of *Cistus* (Fig. 4). Although we obtained limited phylogenetic support, a sister-group relationship between *H. umbellatum* and the white-flowered species of *Cistus* is observed in some reconstructions (Fig. 5). In fact, the three species (*C. clusii*, *C. munbyi*, and *C. libanotis*) of *Cistus* subgenus *Halimioides* are morphologically similar to *Halimium* in terms of leaf shape (linear), sepal number (3), and seed production (oligosperm placenta). *Cistus* subgenus *Halimioides* was recognized by some authors (Demoly and Montserrat, 1993) but not by others (Dansereau, 1939; Dunal, 1824; Willkomm, 1856). None of these taxonomic treatments of *Cistus* (Table 1) is fully congruent with the strict consensus tree of the combined analysis of *trnL-F*, *matK*, and ITS sequences (Fig. 5). The division of *Cistus* into two more subgenera formed by species with purple (subgenus *Cistus*) and white (subgenera Leucocistus) flowers is mostly supported, as *C. parviflorus* appears in all analyses as the only purple-flowered species placed in an otherwise white-flowered lineage. Its distinctiveness has been historically recognized by creating a monotypic, supraspecific taxon (section) for this species as *Ledonella*. The recognition of a group of Canarian species, as a supraspecific taxon (usually called Rhodocistus) within *Cistus* subgenus *Cistus*, accords with the well-diagnosed natural group of long-styled species exclusive to the Canary Islands (Fig. 4). One more supraspecific taxon (section Erythrocistus), consisting of *C. albidus*, *C. creticus*, *C. heterophyllus*, and *C. crispus*, is paraphyletic because the Canarian lineage originated from a most recent common ancestor to only three of them. At a finer level of taxonomic resolution, this study supports present-day delimitation of some species (*C. parviflorus*, *C. salviifolius*, *C. libanotis*, *C. psilosepalus*, *C. ladanifer*, *C. laurifolius*, *C. pouzolzii*, and *C. monspeliensis*). Our population sample, although limited, indicates that neither paraphyly nor polyphyly affect species formation in *Cistus*. One more lineage consisting of the three subspecies of *C. ladanifer*, as circumscribed by Demoly and Montserrat (1993), receives strong support (Fig. 4). However, our phylogenetic hypothesis does not resolve relationships among these subspecies and our data are unable to determine whether populations from southern Portugal should be recognized as *C. ladanifer* subsp. *sulcatus* or as a distinct species (*C. palinhae* Ingram).

4.2. Evolution of morphological characters

Ten morphological characters have been traditionally considered for circumscription of *Cistus*. Data for some of these characters were not available for all species included in the study (Supplementary Table S2), but five are shown mapped on the total-evidence phylogeny: petal color, leaf base, sepal number, style length, and number of fruit valves (Fig. 4). MacClade reconstructions indicate a dynamic course of evolution of morphological characters (Fig. 5). Our phylogenetic hypothesis suggests that purple petals appear to originate twice in the *Halimium–Cistus* assemblage, result partly in agreement with taxonomic treatments since petal color serves to define a natural group of all purple-flowered species, except for *C. parviflorus*. Leaf bases experienced multiple changes not only in *Cistus* lineages, but also in the Cistaceae and potentially within a single species (*C. ladanifer*). Three and five sepals are found across the Cistaceae reflecting multiple shifts in many groups. Five sepals are, however, maintained within the lineage of purple-flowered species. Our data suggest that the four states of style length (sessile, shorter, similar, and longer) did not evolve in a unidirectional manner in either Cistaceae or the *Cistus–Halimium* assemblage (Fig. 5A). Stigmas exceeding stamens occur only in the Canarian species of *Cistus* and appear to have evolved from an ancestor in

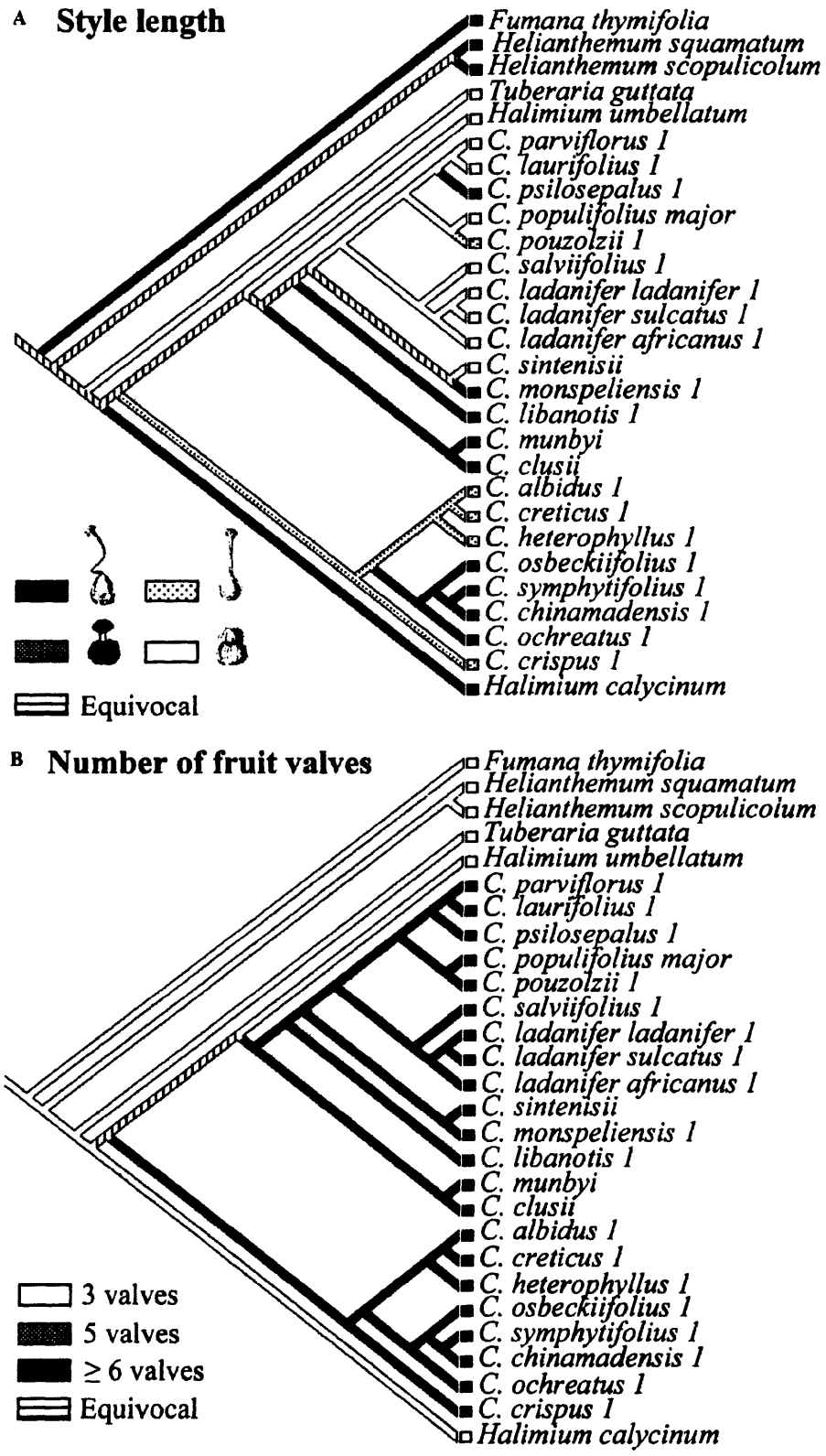

Fig. 5. Hypothesis of character evolution for style length (A) and number of fruit valves (B) using sequences from one individual per taxon. This MP tree of the combined analysis of *trnL-F*, *matK*, and ITS sequences chosen for character reconstruction, onto which the two characters have been mapped, is congruent with the BI tree (see text) and shows "all parsimonius states" as implemented in MacClade (Maddison and Maddison, 1992).

the lineage of purple-flowered species with styles equal in length to the stamens. The optimization of long styles is consistent with an early acquisition of this unique character state, which was then maintained during the course of speciation in the Canary Islands. It is intriguing to interpret prominent stigmas in the Canarian *Cistus* as evolution of a trait related to particular environment conditions of oceanic islands. The occurrence of long styles in other continental Cistaceae indicates recurrent acquisition of this character (Fig. 5A).

Historical reconstruction of the evolution of the number of fruit valves provides evidence of equivocal transition from three valves, consistently displayed in over 180 species of the Cistaceae, to five valves in *Cistus* (Fig. 5B). A further step resulted in the increment of fruit valves to 6–12 exclusively in a single species (*C. ladanifer*). A consistent number of fruit valves is not only exhibited within species of the Cistaceae but also within genera of closely related families (Nandi, 1998). *C. ladanifer* constitutes then a remarkable species model to explore multiplication of fruit valves during the development of the ovary wall. Maximum ITS sequence divergence (0.93% K-2-p distance) of extant subspecies, in comparison to another angiosperms (Richardson et al., 2001), suggests that the multi-valved fruit of *C. ladanifer* evolved following establishment of the Mediterranean climate 2.8 Ma (Suc, 1984) and after plants with 5-valved fruits had been in existence for million of years (see below). Although this character may be subject to phylogenetic processes, variation between 6 and 12 valves should be studied in a broader sense because the highest number of valves appears to be environment dependent in some populations of *C. ladanifer* subsp. *ladanifer* (Narbona, Guzmán, and Vargas, unpublished data).

4.3. Hybridization and evolution in Cistus

Reproductive mechanisms that act to prevent mating occur at the individual level (self-incompatibility) in many species of *Cistus* and *Halimium* (Dansereau, 1940; Herrera, 1992). Consequently, outcrossing favors both inter-individual and inter-specific production of hybrids. Artificial hybridization experiments carried out between 1860 and 1868 by Bornet (Gard, 1910, 1913, 1914) illustrate the facility in which species of *Cistus* generate F_1, F_2, F_3, and F_4 offspring. Bornet successfully undertook crosses between six species of *Cistus* that gave rise to fertile progeny (Gard, 1910). Formation of an intergeneric hybrid between *Cistus* and *Halimium* (x *Halimiocistus*) demonstrates even a wider range of genetic compatibility. Natural hybridization is common as species pairs of *Cistus* occur in sympatry. For instance, 38 natural hybrids between varieties and species were recorded in the field by Dansereau (1940) and 20 inter-specific hybrids of *Cistus* have been described from the Iberian Peninsula (Demoly and Montserrat, 1993; Martín and Guinea, 1949). In light of these results, hybridization was hypothesized as the major mode of evolution in *Cistus* since the early 20th century (Dansereau, 1940; Demoly, 1996). Molecular evidence for hybridization is manifested by (1) nucleotide double-peak patterns (additivity) in 10 ITS sequences, (2) more than two ITS copies of different length size from PCR amplifications of the same DNA samples, and (3) five of the 15 nucleotide additivity sites are found at parsimony-informative positions. This, together with the biparental inheritance of the ITS region, is interpreted as an argument of alternative ITS copies inherited by two or more parental donors (Fuertes et al., 1999), followed by failure to fully homogenize (concerted evolution) multiple ITS copies in the nuclear genome over generations (Rauscher et al., 2002). Apart from success in obtaining artificial inter-species crossings and detecting a molecular pattern of sequence additivity in *Cistus*, the occurrence of hybrid swarms in certain locations of Morocco and Spain (unpublished data) supports the viability of hybridization as an evolutionary mechanism in *Cistus*.

Conflicting signals observed from phylogenies of recombinant, biparental nuclear ribosomal ITS vs non-recombinant, uniparental organelles have typically been interpreted as evidence for extensive reticulation processes at the species level (Wendel and Doyle, 1998). Unfortunately, the limited resolution obtained in the ITS phylogeny precludes detecting fundamental discordance with the plastid phylogeny (Fig. 3). A potential case of speciation by hybridization should be further explored in *C. parviflorus*, as suggested by the detection of sequence additivity (ITS additivity of nucleotides in two sites and different-length sequences in one accession) and the combination of two morphological characters within this species that otherwise define exclusively the two *Cistus* lineages, i.e., purple petals and sessile stigmas. While incongruence between nuclear and organelle genomes may reveal evidence for reticulation, the converse is not necessarily true based only on ITS sequences (Chase et al., 2003). The use of alternative nuclear markers, such as single-copy genes (already in progress), may shed further light on whether balanced concerted evolution of ITS sequences impedes obtaining full-resolved phylogenies (Nieto Feliner et al., 2001).

4.4. Differentiation in the Mediterranean

Cistus exhibits a dominant role in woodland understory and evergreen scrub of the Mediterranean region (Médail and Quézel, 1997). The major center of species diversity is in the western Mediterranean, particularly on both sides of the Strait of Gibraltar (14 of 20 in both Andalusia and northern Morocco (Fig. 1)). The same is true for *Halimium* (the closest genus to *Cistus*), with most species (8 of 10) distributed in this area. Early differentiation of present-day *Cistus* may have occurred

in the western Mediterranean based on the following molecular evidence: (1) the total-evidence phylogeny reveals that the six species of *Cistus* exclusively occurring in the eastern Mediterranean and the Canary Islands (*C. sintenisii* in Albania and Greece; *C. parviflorus* in Greece, Turkey, Italy, Cyprus, and Libya; and *C. chinamadensis*, *C. ochreatus*, *C. osbeckiifolius*, and *C. symphytifolius* in the Canary Islands) do not form basal-most sister groups (Fig. 4); (2) a western-Mediterranean species (*C. crispus*) is sister to the remaining species of the purple-flowered lineage (excluding *C. parviflorus*), as well as two western-Mediterranean species (*C. clusii*, *C. munbyi*) to the white-flowered lineage; (3) the 14 species distributed in the western Mediterranean reach levels of pairwise sequence divergence similar to those within the whole genus (1.62 vs 1.78% in *mat*K; 2.88 vs 3.15% in *trnL-F*; and 4.36 vs 4.86% in ITS (Table 2)). High morphological (taxonomy) and molecular (phylogenetics) divergence in the western Mediterranean and Macaronesia suggests a prime hotspot of diversity not only in *Cistus* but also in disparate angiosperms (Médail and Quézel, 1997). It has been suggested that regional diversity in mediterranean-climatic regions is the product of local diversity and differentiation diversity in relation to environmental heterogeneity (Cowling et al., 1996; Thompson, 2005). *Cistus* species do not fall into this diversity pattern in spite of absence of a long-distance dispersal syndrome (dry capsules and seeds) and environmental specificity referring to acidic and carbonate substrates. In fact, there are no endemics to particular Mediterranean countries and no pattern of geographic cohesion (Fig. 4). Dispersal and colonization of *Cistus* across areas in the Mediterranean basin is inferred to have taken placed successfully after divergence and species formation. Besides wide distribution of most species, the occurrence of circum-Mediterranean species (*C. creticus*, *C. monspeliensis*, and *C. salviifolius*) in the two major lineages supports this view.

The oldest pollen record for Cistaceae (*Cistacearumpollenites*) dates from the Lower Miocene from Czechia (Bohemia) (Konzalova, 1967). This identification should be taken cautiously as there are difficulties in identifying pollen samples at the genus level; identification of species is, however, most reliable once determining ascription to *Cistus* (Ukraintseva, 1991). In contrast, *Cistus* displays an unequivocal shape and number of fruit valves (Supplementary Table S1) in the Mediterranean flora. Fruits in the sedimentary rocks of Germany (Montbauer) and in the amber-bearing sands of the Baltic Sea (Zemland) from the Oligocene (Palibin, 1909) provide reliable evidence for a distribution of *Cistus* not restricted to the present-day Mediterranean region. Objections about inference of centers of origin have been extensively discussed in the past (Bremer, 1992). Despite present-day distribution and diversity of *Cistus* species, paleobotanical data strongly suggest centers of origin for *Cistus* out of the Mediterranean region (as nowadays outlined, Fig. 1), and a time of formation of at least 23 million years ago. We cannot consider calibrated divergence times for *Cistus* because no absolute substitution rate (molecular clock) has been estimated in the Cistaceae. Using both mean values of ITS divergence (4.37×10^{-9} nucleotide substitution/site/year) in angiosperms (Richardson et al., 2001) and the maximum ITS sequence divergence found within *Cistus* (4.86% K-2-p divergence), we infer that differentiation of extant species of *Cistus* might not predate 8–7 Ma. If this result is consistently obtained in future investigations, as increasing the sample and using alternative markers and molecular-clock methods, it would be plausible a hypothesis of that present-day species differentiation occurred much later than the formation of *Cistus*.

4.5. Historical biogeography of Canarian species

Floristic affinities, number of introductions, time of dispersal, and speciation patterns have been the major objectives inferred for Macaronesian plant groups by means of molecular phylogenetics (Carine et al., 2004). The biogeographic results presented in this paper support general patterns of plant colonization in the Canary Islands. The species of *Cistus* endemic to the Canary Islands are imbedded in the purple-flowered lineage in the total-evidence analysis (Fig. 4). Both plastid and nuclear phylogenies reveal a single colonization of *Cistus* in the Canary Islands to account for present-day differentiation into four species (*C. ochreatus*, *C. chinamadensis*, *C. osbeckiifolius*, and *C. symphytifolius*) (Fig. 3). Despite a significant number of phylogenies including Macaronesian taxa, few of them use molecular data from different cellular genomes and, where they have been used, few are congruent with the placement of Canarian lineages (Francisco-Ortega, 2004). We herein provide strong multigenome evidence for a single introduction of purple-flowered *Cistus* in the Canary Islands. Single introductions in Macaronesia appear to be the rule rather than the exception for plant groups consisting of numerous species (Carine et al., 2004; Silvertown, 2004; Vargas, 2005), although topological congruence between organullar and nuclear markers and a larger number of examples are needed. One question that remains to be resolved is whether the occurrence of the white-flowered species *C. monspeliensis* in the Canary Islands and Madeira is the result of natural or human-influenced introduction.

Phylogenetic reconstructions place the Canarian endemics of *Cistus* in a clade with three purple-flowered continental species (*C. albidus*, *C. heterophyllus*, and *C. creticus*). A set of morphological attributes, such as petiolate leaves and a high number of stamens, appear to relate *C. heterophyllus* to the Canarian species (Supplementary Table S2). This species is currently

distributed in the western Mediterranean supporting the close floristic relationship between the Canary Islands and the Mediterranean (Carine et al., 2004), and particularly with north-western Africa since *C. heterophyllus* occurs almost exclusively in Morocco and Algeria. Irrespective of the closest, extant relative of the Canarian lineage, character-state reconstruction using MacClade reveals that the four species endemic to the Canary Islands (*C. ochreatus*, *C. chinamadensis*, *C. osbeckiifolius*, and *C. symphytifolius*) originated from a 5-sepaled, purple-flowered, mid-styled, and 5 fruit-valved ancestor (Figs. 4 and 5). Once *Cistus* colonized and established in the archipelago, speciation took place in conjunction with maintenance of long styles exceeding stamens (Fig. 5A).

Previous allozyme diversity results (Batista et al., 2001) are in agreement with the levels of nucleotide divergence found in the present study, in which *C. symphytifolius* displays the highest levels of K-2-p pairwise divergence with respect to the other Canarian species: 0.32% for ITS (between *C. symphytifolius* 2 and *C. chinamadensis* 1); 0.00% for *matK*; and 0.71% for *trnL-F* (between *C. symphytifolius* and *C. osbeckiifolius*). Additionally, populations of *C. symphytifolius* from different islands display a polyphyletic pattern and the highest levels of nucleotide pairwise divergence for these three markers (unpublished data). Given that Tenerife and Gran Canaria harbor the four species of *Cistus* and the highest levels of molecular diversity, including isozyme and nucleotide divergence, we hypothesize that *Cistus* lineages from these two islands have spawned new lines of evolution via interisland dispersal. Two major evolutionary models have been described to explain speciation of angiosperms in the Canary archipelago: interisland dispersal followed by speciation and intraisland radiation followed by dispersal to similar habitats (Baldwin et al., 1998). The bulk of molecular evidence and present-day distributions suggest extensive interisland dispersal of *C. symphytifolius*, or a closely related ancestor, followed by differentiation of new taxa in some islands (Batista et al., 2001). These conclusions require further intraspecific sampling of *C. symphytifolius* from every island.

Accelerated morphological diversification has been hypothesized for insular plant groups commonly regarded as examples of explosive radiation in which sequence identity is maintained (Baldwin et al., 1998). We do not hypothesize explosive speciation for *Cistus* because of a low number of Canarian species (five including a recently described species by Demoly (2004)) separated by considerable tree branch length with respect to continental species. Levels of molecular divergence of Canarian and their closest continental relatives (0.94% for ITS; 0.47% for *matK*; and 0.71% for *trnL-F*) suggest a relatively old colonization, which contrasts with similar habit (shrubs) as that of their closest relatives in the continent. Differentiation of genera colonizing oceanic islands resulted in tree-like plants in a numerous number of plant groups and remarkable shifts from herbs to a woody condition (Baldwin et al., 1998). Given a considerable time for establishment of *Cistus* in the Canary Islands, it is intriguing to observe neither an increment in size (woodiness) nor occupation of new habitats. Competition-free environments characteristic as oceanic island formation were, however, likely to be rare by the relatively time of *Cistus* establishment, as inferred by low sequence divergence with regard to the origin of the oldest island (Fuerteventura, 20.7 Ma) (Silvertown, 2004). In addition, limited capability of *Cistus* to succeed in different habitats in the continent may be related to failure in exploitation of new, diverse habitats already occupied in oceanic islands. The four species of *Cistus* inhabit Canarian woodlands as understory, and form part of successional stages of Mediterranean and pine tree communities, therefore similar in ecology to their continental congeners (Ceballos and Ortuño, 1976). Lineages of two broom genera (*Adenocarpus*, *Teline*) also exhibit adaptation to woodland understory with no shift in woodiness, limited exploitation of new ecological niches, and similar levels of ITS sequence divergence related to species number (Percy and Cronk, 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2005.04.026.

References

Alfaro, M.E., Zoller, S., Lutzoni, F., 2003. Bayes or bootstrap. A simulation study comparing the performance of bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Mol. Biol. Evol. 20, 255–266.

Álvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.

Arrington, J.M., Kubitzki, K., 2003. Cistaceae. In: Kubitzki, K. (Ed.), The families and genera of vascular plants IV. Flowering Plants. Dicotyledons. Malvales, Capparales and Non-betalain Caryophyllales. Springer, Berlin, pp. 62–70.

Baldwin, B.G., Crawford, D.J., Francisco-Ortega, J., Kim, S.-C., Sang, T., Stuessy, T.F., 1998. Molecular phylogenetic insights on the origin and evolution of oceanic island plants. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer Academic Publishers, Boston, pp. 410–441.

Batista, F., Bañares, A., Caujapé-Castells, J., Carqué, E., Marrero-Gómez, M., Sosa, P.A., 2001. Allozyme diversity in three endemic species of *Cistus* (Cistaceae) from the Canary Islands: intraspecific and interspecific comparisons and implications for genetic conservation. Am. J. Bot. 88, 1582–1592.

Bosch, J., 1992. Floral biology and pollinators of three co-occurring *Cistus* species (Cistaceae). Bot. J. Linn. Soc. 109, 39–55.

Bremer, K., 1992. Ancestral areas: a cladistic reinterpretation of the center of origin concept. Syst. Biol. 41, 436–445.

Carine, M.A., Russell, S.J., Santos-Guerra, A., Francisco-Ortega, J., 2004. Relationships of the Macaronesian and Mediterranean floras: molecular evidence for multiple colonizations into Macaronesia and back-colonization of the continent in *Convolvulus* (Convolvulaceae). Am. J. Bot. 91, 1070–1085.

Ceballos, L., Ortuño, F., 1976. Estudio sobre la vegetación y flora forestal de Las Canarias occidentales. Excelentísimo Cabildo Insular, Santa Cruz de Tenerife, 433pp.

Chase, M.W., Knapp, S., Cox, A.V., Clarkson, J.J., Butsko, Y., Joseph, J., Savolainen, V., Parokonny, A.S., 2003. Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). Ann. Bot. 92, 107–127.

Cowling, R.M., Rundel, P.W., Lamont, B.B., Arroyo, M.K., Arianoutsou, M., 1996. Plant diversity in Mediterranean-climate regions. TREE 11, 362–366.

Dansereau, P.M., 1939. Monographie du genre *Cistus*. Boissiera 4, 1–90.

Dansereau, P., 1940. Études sur les hybrides de Cistes. Ann. Épiphyt. 6 (Ser. 2), 7–26.

Demoly, J.P., Montserrat, P., 1993. *Cistus*. In: Castroviejo, S. et al. (Eds.), Flora Ibérica, vol. 3. Consejo de Investigaciones Cientificas, Madrid, pp. 319–337.

Demoly, J.P., 1996. Les hybrides binaires rares du genre *Cistus* L. (Cistaceae). Anales Jard. Bot. Madrid 54, 241–254.

Demoly, J.P., 2004. Une nouvelle espèce du genre *Cistus* L. (Cistaceae) endémique de l'île de Grande Canarie (Espagne). Acta Bot. Gallica 151, 231–232.

Ducousso, M., Béna, G., Bourgeois, C., Buyck, B., Eyssartier, G., Vincelette, M., Rabevohitra, L., Randrihasipara, L., Dreyfus, B., Prin, Y., 2004. The last common ancestor of Sarcolaenaceae and Asian dipterocarp trees was ectomycorrhizal before the India-Madagascar separation, about 88 million years ago. Mol. Ecol. 13, 231–236.

Dunal, M.F., 1824. Cistineae. In: De Candolle, A.P. (Ed.), Prodromus Systematis Naturalis Regni Vegetabilis I. Treuttel et Würtz, Paris, pp. 263–286.

Ellul, P., Boscaiu, M., Vicente, O., Moreno, V., Rosselló, J.A., 2002. Intra- and interspecific variation in DNA content in *Cistus* (Cistaceae). Ann. Bot. 90, 345–351.

Francisco-Ortega, J., 2004. Origin of the Macaronesian flora and its relationships with the Mediterranean flora. In: IX Meeting on International Organization of Plant Biosystematics. Plant Evolution in Mediterranean Climate Zones, May 16–19, Valencia, Spain.

Fuertes, J., Rosselló, J.A., Nieto, G., 1999. Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). Mol. Ecol. 8, 1341–1346.

Gard, M., 1910. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Premier mémoire: notes inédites et résultats expérimentaux. Ann. Sci. Nat. Ser. 9, 12, 71–115.

Gard, M., 1913. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Deuxième mémoire: les espèces et les hybrides binaires. Beih. Bot. Centralbl. 29, 306–394.

Gard, M., 1914. Recherches sur les hybrides artificiels de Cistes, obtenus par Ed. Bornet. Troisième mémoire: Les hybrides dérivés et les hybrides complexes. Beih. Bot. Centralbl. 31, 373–428.

Grosser, W., 1903. Cistaceae. In: Engler, A. (Ed.), Dans Pflanzenreich 14, Berlin, 161 pp.

Herrera, J., 1986. Flowering and fruiting phenology in the coastal shrublands of Doñana, south Spain. Vegetatio 68, 91–98.

Herrera, J., 1987. Flower and fruit biology in southern Spanish Mediterranean shrublands. Ann. Mo. Bot. Gard. 74, 69–78.

Herrera, J., 1992. Flower variation and breeding systems in the Cistaceae. Plant Syst. Evol. 179, 245–255.

Johnson, L.A., Soltis, D.E., 1994. matK DNA and phylogenetic reconstruction in Saxifragaceae s. str. Syst. Bot. 19, 143–156.

Johnson, L.A., Soltis, D.E., 1998. Assessing congruence: empirical examples from molecular data. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer Academic Publishers, Boston, pp. 297–343.

Kelchner, S.A., 2000. The evolution of non-coding chloroplast DNA and its application in plant systematics. Ann. Mo. Bot. Gard. 87, 482–498.

Konzalova, M., 1967. Paleontologicke zprava klokalite. Archiv Geoindustria, Geofond, Prague.

Maddison, W.P., Maddison, D.R., 1992. MacClade: Analysis of phylogeny and character evolution. Version 3.01. Sinauer Associates, Sunderland, MA.

Martín, M., Guinea, E., 1949. Jarales y jaras (Cistografía Hispánica). Bol. Inst. Forest. Invest. Exp. 49, Madrid, 228pp.

Médail, F., Quézel, P., 1997. Hot-spots analysis for conservation of plant biodiversity in the Mediterranean basin. Ann. Mo. Bot. Gard. 84, 112–127.

Nandi, O.I., 1998. Ovule and seed anatomy of Cistaceae and related *Malvanae*. Plant Syst. Evol. 209, 239–264.

Nieto Feliner, G., Fuertes, J., Rosselló, J.A., 2001. Can extensive reticulation and concerted evolution result in a cladistically structured molecular data set? Cladistics 17, 301–312.

Nylander, J.A.A., 2002. MrModeltest v1.0b. Department of Systematic Zoology, Uppsala University (program distributed by the author).

Palibin, I.V., 1909. Cistaceae. In: Kuznetsov, N., Bush, N., Fomin, A. (Eds.), Flora Caucasica Critica, vol. 3, pp. 117–123.

Percy, D.M., Cronk, Q.C.B., 2002. Different fates of island brooms: contrasting evolution in *Adenocarpus*, *Genista*, and *Teline* (Genisteae, Fabaceae) in the Canary Islands and Madeira. Am. J. Bot. 89, 854–864.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14 (9), 817–818.

Rauscher, J.T., Doyle, J.J., Brown, H.D., 2002. Internal transcribed spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (Leguminosae) polyploid complex. Mol. Ecol. 11, 2691–2702.

Richardson, J.E., Pennington, R.T., Pennington, T.D., Hollingsworth, P.M., 2001. Rapid diversification of a species-rich genus of neotropical rain forest trees. Science 293, 2242–2245.

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Roy, J., Sonié, L., 1992. Germination and population dynamics of *Cistus* species in relation to fire. J. Appl. Ecol. 29, 647–655.

Saenz, C., 1979. Pollen morphology of Spanish Cistaceae. Grana 18, 91–98.

Salamin, N., Chase, M.W., Hodkinson, T.R., Savolainen, V., 2003. Assessing internal support with large phylogenetic DNA matrices. Mol. Phylogenet. Evol. 27, 528–539.

Savolainen, V., Fay, M.F., Albach, D.C., Backlund, A., van der Bank, M., Cameron, K.M., Johnson, S.A., Lledó, M.D., Pintaud, J.C., Powell, M., Sheahan, M.C., Soltis, D.E., Soltis, P.S., Weston, P., Whitten, W.M., Wurdack, K.J., Chase, M.W., 2000. Phylogeny of the eudicots: a nearly complete familial analysis based on *rbcL* gene sequences. Kew Bulletin 55, 257–309.

Silvertown, J., 2004. The ghost of competition past in the phylogeny of island endemic plants. J. Ecol. 62, 168–173.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49 (2), 369–381.

Soltis, D.E., Soltis, P.S., Chase, M.W., Mort, M.E., Albach, D.C., Zanis, M., Savolainen, V., Hahn, W.H., Hoot, S.B., Fay, M.F., Axtell, M., Swensen, S.M., Prince, L.M., Kress, W.J., Nixon, K.C., Farris, J.S., 2000. Angiosperm phylogeny inferred from 18S rDNA, *rbcL*, and *atpB* sequences. Bot. J. Linn. Soc. 133, 381–461.

Soriano, I., 2002. Cistaceae. In: Valdés, B., Rejdali, M., Achhal, A., Jury, J.L., Montserrat, J.M. (Eds.), Checklist of Vascular Plants of N Morocco with Identification Keys, vol. 2. Consejo Superior de Investigaciones Cientificas, Madrid, pp. 196–200.

Spach, E., 1836. Conspectus monographiae Cistacearum. Ann. Sci. Nat. Bot. 6, 357–375.

Suc, J.P., 1984. Origin and evolution of the Mediterranean vegetation and climate in Europe. Nature 307, 429–432.

Sun, Y., Skinner, D.Z., Liang, G.H., Hulbert, S.H., 1994. Phylogenetic analysis of *Sorghum* and related taxa using Internal Transcribed Spacer of nuclear ribosomal DNA. Theor. Appl. Genet. 89, 26–32.

Swofford, D.L., 1999. PAUP*: Phylogenetic Analysis Using Parsimony. Version 4.07b. Sinauer Associates, Publishers, Sunderland, MA.

Taberlet, I., Gielly, L., Pautou, G., Bouvet, J., 1991. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol. Biol. 17, 1105–1109.

Talavera, S., Gibbs, P.E., Herrera, J., 1993. Reproductive biology of *Cistus ladanifer* (Cistaceae). Plant Syst. Evol. 186, 123–134.

Templeton, A.R., 1983. Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. Evolution 37, 221–244.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25 (24), 4876–4882.

Thompson, J.D., 2005. Plant Evolution in the Mediterranean. Oxford University Press, Oxford 288 pp.

Trabaud, L., Renard, P., 1999. Do light and litter influence the recruitment of *Cistus* spp. stands? Israel J. Plant Sci. 47, 1–9.

Ukraintseva, V.V., 1991. The palynological study of the genus *Cistus*. Botanicheskii Zhurnal 76, 979–985 [In Russian].

Vargas, P., 2005. Are Macaronesian islands refugia of relict plant lineages?: a molecular survey. In: Weiss, S.J., Ferrand, N. (Eds.), Phylogeography in Southern European Refugia: Evolutionary Perspectives on the Origins and Conservation of European Biodiversity (in press).

Warburg, E.F., 1968. *Cistus* L. In: Tutin, T.G., Heywood, V.H., Burges, N.A., Moore, D.M., Valentine, D.H., Walters, S.M., Webb, D.A. (Eds.), Flora Europaea, vol. 2. Cambridge University Press, Cambridge, pp. 282–285.

Wendel, J.F., Doyle, J.J., 1998. Phylogenetic incongruence: window into genome history and molecular evolution. In: Soltis, D.E., Soltis, P.S., Doyle, J.J. (Eds.), Molecular Systematics of Plants II DNA Sequencing. Kluwer Academic Publishers, Boston, pp. 265–296.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M., Geldfand, D., Sninsky, J., White, T. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, pp. 315–322.

Willkomm, M., 1856. Cistinearum orbis veteris descriptio monographica. Icones et Descriptiones Plantarum vol. II.